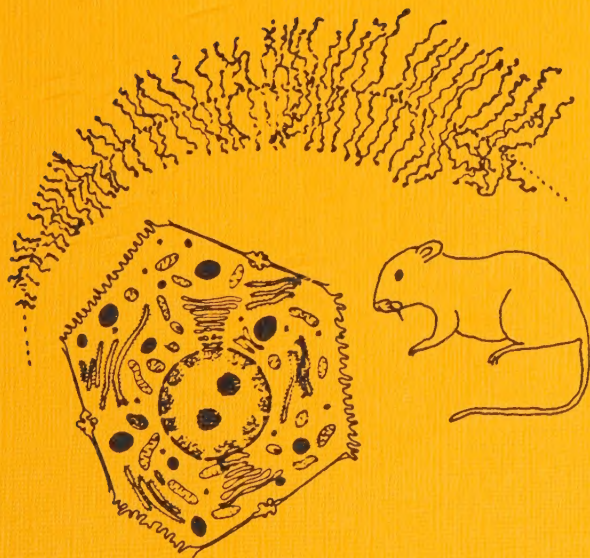


RNA SYNTHESIS DURING LIVER REGENERATION
AND
MODULATION OF RNA PRECURSOR METABOLISM
DURING HEPATOMA GROWTH

Inga Ljungqvist



LUND 1983

RNA SYNTHESIS DURING LIVER REGENERATION
AND
MODULATION OF RNA PRECURSOR METABOLISM
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av

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DOKUMENTTITEL OCH UNDERTITEL RNA synthesis during liver regeneration and modulation of RNA precursor metabolism during hepatoma growth.			
SAMMANFATTNING RNA synthesis in regenerating mouse liver was studied by incorporation of [methyl- ¹⁴ C]methionine and [¹⁴ C]orotic acid <i>in vivo</i> and by determination of RNA polymerase activities in liver nuclei <i>in vitro</i> . Some characteristics related to the synthesis of RNA during normal and neoplastic growth are discussed. The modulating effect of thymidine on the metabolism and toxicity of the pyrimidine 5-fluorouracil (5-FU) was studied <i>in vivo</i> and <i>in vitro</i> . Incorporation of methionine and RNA polymerase activities indicated an increased synthesis of rRNA at 24h and 48h. A decreased synthesis of hn RNA was observed at these times after partial hepatectomy. Total transcription was increased by 32% at 24h. Thus, the increase in transcriptional activity is not as accentuated in regenerating mouse liver as in regenerating rat liver. Incorporation of [¹⁴ C]orotic acid resulted in an increased specific radioactivity of total RNA at 24h. There was a difference in the utilization of orotic acid and uridine between normal, resting liver and hepatoma AH 130. Thymidine potentiated the antitumour effect of 5-FU on hepatoma AH 130. The enhanced cytotoxic effect of 5-FU on tumour cells <i>in vivo</i> was accompanied by an increased incorporation of the substance into tumour RNA. In hepatocytes the catabolism of 5-FU was great, whereas in hepatoma cells no catabolism of 5-FU could be demonstrated. The enhanced incorporation of 5-FU into tumour RNA <i>in vivo</i> after pretreatment with thymidine, was not related to local effects on the tumour cells but rather to an increased bioavailability of the drug. Coadministration of thymidine <i>in vivo</i> resulted in increased but also modulated host toxicity of 5-FU.			
NYCKELORD RNA biosynthesis; RNA polymerase; liver regeneration; hepatoma; pyrimidine metabolism; uridine; orotic acid; 5-fluorouracil; rat; mouse.			
DOKUMENTTITEL OCH UNDERTITEL – SVENSK ÖVERSÄTTNING AV UTLÄNDSK ORIGINALTITEL RNA-syntes i regenererande lever; och inducerade förändringar i metabolismen av en RNA-prekursor, studerade under lever-tumör-tillväxt.			
TILLÄMPNINGSGRÄNS Denna undersökning är ett led i att karaktärisera normal tillväxt, i detta fallet tillväxten i muslever efter partiell hepatektomi. Det är av intresse att studera likheter och skillnader mellan normal tillväxt och tumör-tillväxt. 5-Fluorouracil har ett medicinskt tillämpningsområde inom behandlingen av vissa tumörer, men i denna undersökning har metabolismen av 5-FU studerats på råttor.			
NYCKELORD RNA-syntes; RNA-polymeras; lever-regeneration; lever-tumör; pyrimidin-metabolism; uridin; orotat; 5-fluorouracil; råttor; mus.			
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RNA SYNTHESIS DURING LIVER REGENERATION
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DURING HEPATOMA GROWTH

by

INGA LJUNGQUIST

LUND 1983



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This thesis is based on the following papers and on some additional unpublished observations:

- I Lewan, L., Petersen, I. and Yngner, T. (1975)
Incorporation of orotic acid into nucleotides and RNA in mouse organs during 60 minutes.
Hoppe-Seyler's Z. Physiol. Chem. 356, 425-429.
- II Yngner, T., Petersen, I. and Lewan, L. (1977)
Metabolism of [5-³H]uridine in mouse and specificity for labeling of liver ribonucleic acid.
Int. J. Biochem. 8, 395-401.
- III Ljungquist, I. and Aström, S. (1983)
Effects of partial hepatectomy on RNA polymerase activities in mouse liver.
Int. J. Biochem. (in press).
- IV Ljungquist, I., Yngner, T., Lewan, L. and Engelbrecht, C. (1983)
Ribonucleic acid synthesis of regenerating mouse liver evaluated by incorporation of [methyl-¹⁴C]methionine and [¹⁴C]orotic acid and by determination of RNA polymerase activity.
(submitted for publication).
- V Engelbrecht, C., Ljungquist, I., Lewan, L. and Yngner, T. (1983)
Modulation of 5-fluorouracil metabolism by thymidine. *In vivo* and *in vitro* studies on RNA-directed effects in rat liver and hepatoma.
Biochem. Pharmacol. (in press).

In the text the papers will be referred to by their Roman numerals.

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INTRODUCTION

Liver regeneration

Regenerating liver after partial hepatectomy is an often used model for the study of rapid proliferation (Bresnick, 1971; Bucher & Malt, 1971; Lewan *et al.*, 1977). It is useful as a control tissue when neoplastic growth is examined (Weber *et al.*, 1965; Lea *et al.*, 1974; Duceman & Jacob, 1980). There is often the question of whether the biochemical characteristics obtained during tumour growth are related to the neoplasia itself or whether they can be ascribed to rapid proliferation as such.

Various systems have been used in the attempts to find a comparative model for the study of growth in normal tissues. Neonatal liver and kidney after unilateral nephrectomy are models being employed. However, since the careful description of the method for partial hepatectomy in the rat (Higgins and Anderson, 1931), regenerating liver has been the most common model.

Partial hepatectomy, according to this method, is carried out by resection of the two main lobes of the liver, comprising about two thirds of the total organ. The resultant compensatory hypertrophy and hyperplasia of the residual lobes continues until the original liver mass is restored. Environmental factors as well as age of animal and amount of liver excised influence the rate of regeneration in the remaining lobes (Bucher, 1963). Under defined conditions partial hepatectomy induces reproducible, time-dependent biochemical and morphological events in the residual part of the liver.

One biochemical characteristic is the increased incorporation of thymidine into DNA starting at 16 to 18 h after partial hepatectomy in the rat. DNA synthesis is maximal at 20 to 24 h, and mitosis follows 6 to 8 h later (Grisham, 1962). The regenerative process of mouse liver is

somewhat slower with the maximum incorporation of thymidine into DNA at 36 h (Lewan *et al.*, 1975). The prolongation of the regenerative process found in mouse liver, compared to rat liver, has been ascribed to differences in energy metabolism between the species (Yngner *et al.*, 1980).

During an initial period of approximately 16 h in the rat and 24 h in the mouse the cells are changed from a non-proliferative state to one of active growth. After approximately two weeks the liver mass is again in balance and is not further increased. Thus, regenerating liver after partial hepatectomy provides the possibility of studying both the mechanisms for initiation of rapid proliferation and also the mechanisms for the control of normal growth.

The initial phase of liver regeneration is characterized by an increase in RNA synthesis. Enhanced RNA synthesis, determined by incorporation of labelled pyrimidine precursors, is observed as early as 3 to 6 h after partial hepatectomy in the rat (Bucher & Swaffield, 1969), and the maximal rate of RNA synthesis occurs at 12 to 30 h (Bresnick, 1971).

Alterations in gene function involve the appearance of new, short-lived species of RNA, which can be compared with RNA species associated with rapid cell proliferation in embryonic liver (Church & McCarthy, 1967). Any new genetic information regarding liver proteins must be transcribed into mRNA, and ribosomes are required for subsequent translation of mRNA. Ribosomal RNA accounts for approximately 80% of the total RNA present in cells, and there is a great requirement for synthesis of rRNA during proliferation.

DNA dependent RNA polymerases

Transcription of DNA into RNA is catalyzed by DNA dependent RNA polymerases. Eukaryotes contain several distinct RNA polymerases, which are involved in the synthesis of different species of RNA. For review

articles on the structure and function of eukaryotic RNA polymerases, see Chambon, 1975, and Jacob & Rose, 1980.

The nuclear enzymes were numbered RNA polymerase I, II and III in their order of elution by chromatography with increasing ionic strength. Enzyme I is located in the nucleolus and is responsible for the synthesis of preribosomal RNA (pre rRNA). Polymerase II is located in the nucleoplasm and produces a population of RNA molecules of various molecular weights, called heterogenous nuclear RNA (hn RNA). Some of these molecules are precursors to messenger RNA (m RNA). RNA polymerase III is also active in the nucleoplasm and catalyzes the synthesis of precursor transfer RNA (pre tRNA) and 5S RNA, which is an RNA species found in ribosomes. The RNA polymerases have also been classified according to their sensitivity to α -amanitin (Kedinger, 1970). RNA polymerase I is not affected by the toxin, polymerase II is highly sensitive, whereas polymerase III is sensitive only to high doses of α -amanitin.

In Papers III and IV the differing sensitivity of the polymerases to α -amanitin was utilized to distinguish between RNA polymerase II activity, which was inhibited by α -amanitin at a concentration of 1.4 $\mu\text{g/ml}$, and RNA polymerase I plus III activity, which was unaffected by the toxin at the given concentration.

Each of the RNA polymerases exists in two functional states. Either the enzyme is active in transcribing endogenous chromatin template (engaged or chromatin-bound) or it is inactive towards the template (free enzyme) (Yu, 1974).

In Paper III the chromatin-bound RNA polymerase activity was studied in liver nuclei. The activity of free RNA polymerase was determined after inhibition of the endogenous template activity by actinomycin D and addition of the synthetic template poly[d(A-T)]. Actinomycin D exerts its inhibitory action on transcription by intercalating between two G-C base

pairs of DNA and binding to the guanosine residue (Sobell & Jain, 1972). Transcription of poly[d(A-T)], with no guanosine present, can thus proceed without inhibition.

Processing of ribosomal RNA

The transcript from RNA polymerase I, pre rRNA, has a sedimentation coefficient of 45S and undergoes a series of maturation processes to become 18S, 28S and 5.8S in their final forms in the ribosome. Considerable spacer regions are eliminated during ribosome maturation, and a number of secondary modifications take place. Modification of the RNA molecule starts rapidly, and more than one hundred methyl groups are added to the newly synthesized 45S RNA (Maden & Salim, 1974). All methylation sites are confined to the ribosomal sequences, and no methylation occurs on the spacer regions. All sites except for about six are on the 2'-hydroxyl groups of the ribose residues of the molecule to prevent enzymatic cleavage (Maden & Salim, 1974; Maden, 1976). Late during ribosome maturation a few base methylations occur to stabilize the conformation of the mature RNA (Engel & von Hippel, 1974).

The methyl groups are derived from methionine. In Paper IV [methyl-¹⁴C]methionine was used as methyl donor in order to obtain incorporation into RNA, after which rRNA was isolated and the specific radioactivity determined.

Incorporation of radioactively labelled pyrimidine precursors

The RNA transcript is synthesized by incorporation of the four ribonucleotide triphosphates ATP, GTP, UTP and CTP. In studies of RNA synthesis, radioactively labelled pyrimidine precursors are used for labelling of UTP and CTP and subsequently of RNA. Orotic acid is a precursor of common use for labelling of rat liver RNA. It is incorporated

along the *de novo* pathway for pyrimidine synthesis (Fig. 1). In Papers I and IV [^{14}C]orotic acid was utilized for labelling of RNA *in vivo*. In Paper II labelling of RNA was obtained by incorporation of [^3H]uridine. This precursor is incorporated along the salvage pathways (Fig. 1). In Papers III and IV liver nuclei were incubated with [^3H]UTP *in vitro* in order to determine RNA polymerase activities.

In Paper V the modified pyrimidine 5-fluorouracil (5-FU) was utilized in studies of rat liver and hepatoma *in vivo* and *in vitro*. The substance is metabolized in analogy with uracil, i.e. along the salvage pathways, but the presence of a fluoro atom instead of hydrogen in the 5-position prevents the methylation of dUMP (deoxyuridine-monophosphate), and the supply of dTTP (thymidine-triphosphate) for DNA synthesis is depleted. Furthermore, FUTP becomes incorporated into RNA, and the fluoro atom interferes with maturation and function of RNA.

Aims of the investigation

To determine the optimal conditions for examination of RNA synthesis in mouse liver by use of radioactively labelled pyrimidines, the efficiency for labelling of RNA by orotic acid (I) and uridine (II) was studied. Little information is available concerning the pyrimidine metabolism in the mouse. The metabolism of the precursors was therefore examined in some detail. The influence on labelling of RNA of different routes of administration of the precursor was also studied (I).

RNA synthesis and pyrimidine metabolism in regenerating mouse liver was then studied in this laboratory, according to the conditions defined in Papers I and II. These investigations revealed no increased incorporation of orotic acid into RNA, UTP or total acid soluble fraction at either 6 h or 24 h after partial hepatectomy (Yngner *et al.*, 1979a; Yngner *et al.*, 1980). Nor was there an increased incorporation of uridine into UTP or

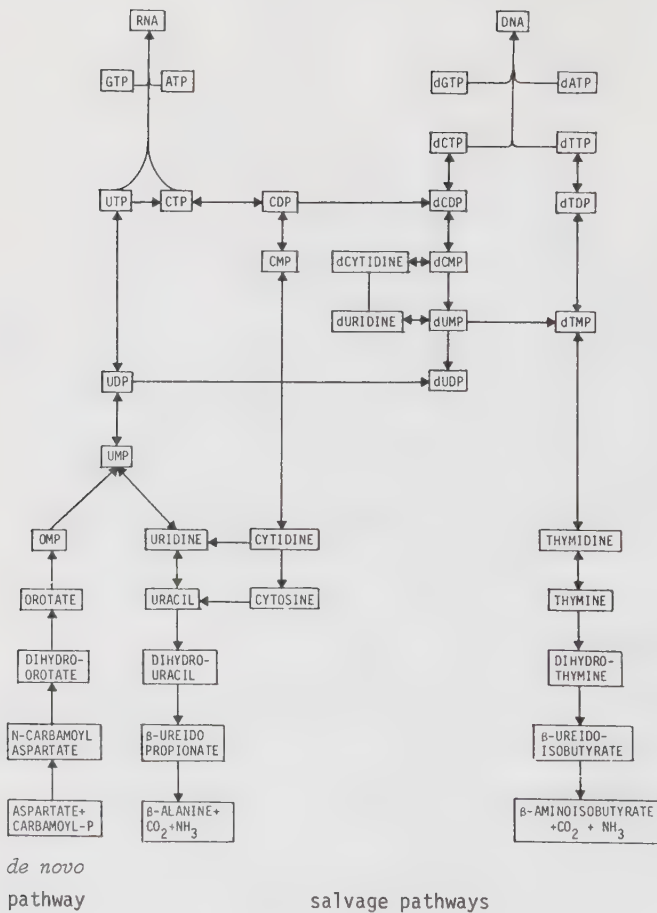


Figure 1.

Pyrimidine metabolism. *De novo* and salvage pathways for incorporation into RNA and DNA.

RNA of regenerating mouse liver despite an increased uptake of the precursor into the total acid soluble fraction (Yngner *et al.*, 1979b).

The fact that no increased incorporation into RNA of either orotic acid or uridine was observed in regenerating mouse liver may be interpreted as indicating that there is no increased RNA synthesis. However, there is obviously a rise in the RNA content during the regenerative process in mouse liver (Lewan, 1972). Thus, either transcription must be increased, though it was not detected by use of labelled orotic acid or uridine, or a decreased degradation of RNA must occur. In Papers III and IV the question of a possible increase in transcription was examined, and RNA synthesis was determined by different methods.

In Paper V rats bearing hepatoma AH 130 were treated with the substituted pyrimidine 5-fluorouracil (5-FU). This substance is used clinically as an anti-tumour agent with both DNA-directed and RNA-directed effects. It has been reported that addition of thymidine leads to enhanced cytotoxicity of 5-FU (Santelli *et al.*, 1978; Spiegelman *et al.*, 1980a; Fried *et al.*, 1981), and this effect has been correlated with an increased incorporation of 5-FU into RNA (Spiegelman *et al.*, 1980a; Young *et al.*, 1981). The modulating effect of thymidine on the metabolism and toxicity of 5-FU was studied *in vivo* and *in vitro* (V).

RNA SYNTHESIS DURING LIVER REGENERATION

Efficiency of orotic acid and uridine for labelling of RNA in normal liver

Orotic acid and uridine were readily taken up into the acid soluble fraction and incorporated into UTP and RNA of normal mouse liver (I and II). Thus, both the *de novo* pathway and the salvage pathways seem to be

effectively utilized for the synthesis of uridine nucleotides in this tissue.

In rat liver the uptake of orotic acid is even more efficient than in mouse liver (Yngner *et al.*, 1979a). After administration of labelled orotic acid to normal rats, 45-50% of the administered dose is recovered in the liver (Bucher & Swaffield, 1966). In Paper I, the acid soluble fraction of mouse liver contained about 22% of the administered dose. In these investigations orotic acid was administered by intraperitoneal and intravenous injection. Also after oral feeding of orotic acid a greater percentual distribution to the liver is observed in the rat than in the mouse (Durschlag & Robinson, 1980). The precursor is effectively incorporated into UTP and RNA of rat liver (Bucher & Swaffield, 1969). Thus, in rat liver the *de novo* pathway seems to be effectively utilized for synthesis of UTP.

Uridine was also taken up into the acid soluble fraction of rat liver (II), but this precursor was not utilized for synthesis of RNA as efficiently in rat liver as in mouse. The higher activity of the salvage pathway for UTP synthesis in mouse liver (II) may be explained by the fact that uridine kinase is more active in mouse liver compared to rat liver (Reichard & Sköld, 1958).

The rate of precursor incorporation was also influenced by the route of administration (I). A more rapid distribution of the precursor in the body was found after intravenous injection than after intraperitoneal injection (Yngner *et al.*, 1975). Injections into the tail vein were complicated by the fact that unabsorbed isotope remained in the tail of the animal for as long as 60 min (Yngner *et al.*, 1975). In our hands intraperitoneal injection was a more reproducible method, and the relatively slow distribution of the precursor seemed well suited to

analysis of incorporation into nucleotides and RNA. This route of administration of precursors was used in the subsequent studies.

Increased synthesis of ribosomal RNA in regenerating liver

The synthesis of rRNA was studied by use of [methyl- ^{14}C]-labelled methionine (IV). One advantage of using methionine instead of a pyrimidine precursor is that its incorporation into RNA is not influenced by possible changes in size of the pyrimidine pools.

Labelled methyl-groups were incorporated into RNA, after which rRNA was isolated and the specific radioactivity of rRNA determined. Incorporation into regenerating mouse liver was compared to the values of liver from sham-operated and unoperated animals. There was an increased specific radioactivity of rRNA of regenerating liver at both 24 h and 48 h. The increase was 263% at 24 h and 103% at 48 h compared to the values of sham-operated controls. Compared to unoperated controls the increase was even more pronounced.

These results indicated an enhanced synthesis of rRNA during mouse liver regeneration. To further examine the synthetic rate of rRNA after partial hepatectomy RNA polymerase activities were determined (III and IV).

The enzyme activity of chromatin-bound RNA polymerase I plus III showed an increase of 57% and 51% over the values of sham-operated controls at 24 h and 48 h respectively (III and IV). Since RNA polymerase III makes up only approximately 17% of the I plus III activity (Tata & Baker, 1978), the results most certainly reflect an increase in the synthesis of rRNA.

In regenerating liver chromatin-bound RNA polymerase I plus III accounted for a greater percentual fraction than in control liver (III).

In normal liver and liver from sham-operated animals RNA polymerase I plus III accounted for 62-65% of the total activity, whereas in regenerating liver these enzymes together accounted for almost 80%. This is in agreement with the enzyme changes found during regeneration of rat liver (Table 1).

Table 1.

Changes in the relative proportions of the chromatin-bound RNA polymerase activities following partial hepatectomy in mouse and rat.

	Chromatin-bound RNA polymerase I plus III in percent of total RNA polymerase activity	
	mouse liver (24 h)	rat liver (18 h)
Sham-operated control	65%	53% ^a
Partial hepatectomy	78%	70% ^a

^a)Organtini *et al.*, 1975.

The rise in RNA polymerase I activity during mouse liver regeneration is in accordance with several observations on different tissues after varying physiological stimuli. In regenerating rat liver RNA polymerase I activity is enhanced 18 h after partial hepatectomy (Organtini *et al.*, 1975; Yu, 1975; Duceman & Jacob, 1980). Hydrocortisone stimulates RNA polymerase I activity in rat liver (Sajdel & Jacob, 1971), and the same effect is found for the stimulation of testosterone on mouse kidney (Jänne *et al.*, 1976).

After stimulation of RNA synthesis in rat liver by hydrocortisone, the increased RNA polymerase activity is exclusively in polymerase I (Sajdel & Jacob, 1971). In regenerating rat liver, however, an increase is also documented for RNA polymerase II and III, though the most pronounced increase is in polymerase I (Duceman & Jacob, 1980).

Synthesis of hnRNA in regenerating liver

In regenerating mouse liver no increase in chromatin-bound RNA polymerase II activity could be demonstrated at 24 h or 48 h (III). Instead a decrease was found at both 24 h and 48 h compared to sham-operated controls. The possibility cannot be excluded, however, that an increased transcription had already taken place during the regenerative period up to 24 h and was not detected by studying the conditions at 24 h and 48 h after partial hepatectomy.

Some mRNA species are transcribed at a much higher rate in regenerating liver than in normal, resting liver. The synthesis of histones, e.g., is correlated to DNA synthesis (Kuehl, 1979) and should be increased at both 24 h and 48 h in regenerating mouse liver. Thus, during the regenerative period when RNA polymerase II activity was found to be depressed, the synthesis of some mRNA species is likely to be increased. These facts indicate that the synthesis of a considerable proportion of liver proteins, found under normal conditions in mouse liver, must be depressed.

Total transcription in regenerating liver

Total chromatin-bound RNA polymerase activity of regenerating mouse liver was slightly elevated at 24 h (IV). The increase was approximately 30%, which is a very low value in comparison with what has been demonstrated in the rat (Table 2).

Free RNA polymerase activities

The increase in chromatin-bound RNA polymerase I activity during mouse liver regeneration was not accompanied by a similar rise in free RNA polymerase I activity. Instead, after partial hepatectomy, the

Table 2.

Effect of partial hepatectomy on total chromatin-bound RNA polymerase activity in mouse and rat liver.

	Chromatin-bound RNA polymerase activity	
	mouse liver (24 h)	rat liver (17-18 h)
Sham-operated control	100%	100%
Partial hepatectomy	132%	219% ^a , 241-280% ^b

RNA polymerase activity after partial hepatectomy was expressed as a percentage of sham-operated controls.

^a)Organtini *et al.*, 1975

^b)Yu, 1975

activities of free RNA polymerase I plus III and II were at the same level or beneath the level of sham-operated controls (III).

In contrast to these results on mouse liver, the preferential increase in RNA polymerase I plus III activity, expressed by chromatin-bound RNA polymerase in regenerating rat liver, is accompanied by a selective stimulation also of free RNA polymerase I plus III. Both free and chromatin-bound activities revealed an increase of 100-200% (Yu, 1975). Thus, in regenerating rat liver there exists an equilibrium between the free enzyme and the chromatin-bound enzyme in the nuclei, which cannot be demonstrated in mouse liver.

In other proliferating systems, besides regenerating mouse liver, the two populations of RNA polymerases have been shown to respond independently of each other. Systems, where the free RNA polymerase activity responds independently of the chromatin-bound activity, are for example lymphocytes stimulated by PHA (Tillyer & Butterworth, 1978) and the stimulation of dormant *Artemia* (Hentschel & Tata, 1977).

Influence of the operation procedure

Anaesthesia and/or surgery had an effect on the synthesis of RNA, as revealed by the sham-operated controls. At 24 h after operation a depression was seen in chromatin-bound RNA polymerase I plus III and II activity. At 48 h, however, the activities were completely restored to the levels revealed by the unoperated controls. Free RNA polymerase activities were influenced in the same direction as the chromatin-bound activities by the operation procedure, but these RNA polymerase activities were not recovered by 48 h.

An effect of anaesthesia and/or surgery was also found on the incorporation of orotic acid. At 24 h after operation the incorporation of the precursor into the total acid soluble fraction and into RNA was decreased compared to the unoperated controls. At 48 h, however, a depression was no longer found.

Thus, at 24 h an effect of the operation procedure was obvious, whereas at 48 h the animals had recovered from the operation, and most parameters studied in this investigation were restored to the levels revealed by the unoperated controls.

Uptake of orotic acid in regenerating liver and hepatoma

During rat liver regeneration there is an increased uptake of orotic acid (Ord & Stocken, 1972). In mouse liver, however, no such increase in the incorporation of orotic acid into the acid soluble fraction could be demonstrated at 24 h after partial hepatectomy (IV, Yngner *et al.*, 1979a; Yngner *et al.*, 1980). At 48 h there even was a decreased incorporation of orotic acid compared to sham-operated controls (IV). Thus, paralleled to increased mitotic activity a decreased uptake of orotic acid was observed.

A difference also exists in the utilization of orotic acid between normal, resting liver and hepatoma of the rat. Orotic acid is not incorporated into hepatoma RNA to any great extent (Weber *et al.*, 1965; Matsudaira *et al.*, 1968; Lea *et al.*, 1974). During development from normal liver to neoplastic liver the significance of the incorporation of orotic acid has diminished, and uridine is a much more efficient precursor for incorporation into hepatoma RNA. This phenomenon is illustrated by host liver and hepatoma AH 130 in Table 3. The low degree of utilization of orotic acid for RNA synthesis is not restricted to tumours of hepatic origin but may be a more general property of tumour tissues (Lea *et al.*, 1976; Wohlhueter *et al.*, 1980). In rat hepatoma it has been suggested that an impaired transport of orotic acid across the membrane may explain the low rate of incorporation of the precursor into the acid soluble fraction and into RNA when compared to regenerating liver and to host liver (Weber *et al.*, 1965; Matsudaira *et al.*, 1968; Lea *et al.*, 1974).

Table 3.

Specific radioactivity of total RNA in rat liver and hepatoma AH 130 after labelling with [^{14}C]orotic acid and [^3H]uridine.

	Specific radioactivity of RNA	
	orotic acid	uridine
Liver	3 990 $\pm$ 200	588 $\pm$ 122
Tumour	523 $\pm$ 47	16 600 $\pm$ 2 300

On day 6 of tumour growth rats were given an i.p. injection of [$6\text{-}^{14}\text{C}$]orotic acid (3.5 μCi , 61 mCi/mmol) or [$6\text{-}^3\text{H}$]uridine (100 μCi , 24 Ci/mmol). The animals were killed 30 min after the administration of labelled precursor, and tissues were sampled in accordance with paper V. RNA was determined according to Munro & Fleck (see Materials and Methods, Paper V). Each value represents mean $\pm$ SEM of duplicate samples from four animals.

The increased utilization of uridine for labelling of RNA in hepatoma compared to normal liver may be explained by the fact that the capacity for degradation of uridine is very low in hepatoma, whereas in normal liver the catabolism of uridine is great (Queener *et al.*, 1971).

Incorporation of orotic acid into RNA of regenerating liver

In Papers III and IV it was shown that there was an increased synthesis of rRNA during mouse liver regeneration. Furthermore, there was an increase of approximately 30% in total transcription at 24 h (IV). Nevertheless, at evaluation of RNA synthesis by incorporation of orotic acid no increased synthesis has been indicated (Yngner *et al.*, 1979a; Yngner *et al.*, 1980).

In kidney after unilateral nephrectomy the labelling of RNA also seems to differ between mouse and rat. As in regenerating rat liver there is an increased incorporation of orotic acid in rat kidney RNA during compensatory hypertrophy (Cortes *et al.*, 1976). In mouse kidney after unilateral nephrectomy, however, no increased incorporation of uridine or orotic acid into RNA has been demonstrated (Hill *et al.*, 1974).

Since the greatest increase in transcription was revealed by rRNA in regenerating mouse liver (III, IV), incorporation of orotic acid was examined with a relatively long labelling time, 120 min, in order to obtain incorporation into this class of RNA (IV). Under these conditions an increased specific radioactivity of rRNA relative to sham-operated controls was found 24 h after partial hepatectomy. Relative to unoperated controls, however, the specific radioactivity was not increased at 24 h. In regenerating liver at 48 h the specific radioactivity of rRNA was decreased both compared to sham-operated and unoperated controls. The low labelling at 48 h seemed to reflect changes in the uptake of the precursor (IV). When

differences in the uptake of the precursor were taken into account the relative incorporation into RNA of regenerating liver exceeded that of sham-operated controls at both 24 h and 48 h. However, any changes in the specific radioactivity of UTP during the pulse period influence the incorporation into RNA, and the net radioactivity of the acid soluble fraction and UTP after labelling for 120 min cannot be used for accurate correction of the incorporation into RNA.

In rat liver the UTP pool is known to expand early after partial hepatectomy (Bucher & Swaffield, 1969; Glazer, 1973), which leads to a decreased specific radioactivity of UTP in regenerating liver compared to the values of sham-operated controls. At examination of the UTP pool during mouse liver regeneration, however, no changes were found in the total UTP pool that could account for the fact that incorporation of orotic acid into RNA was not increased (Yngner *et al.*, 1979a).

The evaluation of RNA synthesis by incorporation of labelled pyrimidine precursors is further complicated by the fact that there seems to be a compartmentation of the nucleotide pools, i.e. only a small part of the total cellular UTP is the immediate source for RNA synthesis (Wiegers *et al.*, 1976; Genchev *et al.*, 1980; Keppler & Holstege, 1982). It has been proposed that hn RNA and pre rRNA are synthesized from partly independent nuclear pyrimidine pools (Genchev *et al.*, 1980). This suggestion is based on the findings that hn RNA is preferentially labelled by orotic acid whereas the synthesis of pre rRNA is rather supplied by UTP labelled by incorporation of uridine. Since there is a considerable increase in the synthesis of hn RNA during rat liver regeneration (Duceman & Jacob, 1980) but not during mouse liver regeneration at 24 h and 48 h (III), the utilization of orotic acid for RNA synthesis should not be expected to be the same during regeneration in these two species.

Concluding remarks on characteristics related to the synthesis of RNA in liver and hepatoma.

The characteristics for normal growth regarding transcription and RNA precursor utilization vary between different species and also between organs within the same species. Regenerating mouse and rat liver utilize orotic acid to a different extent, which may depend on the efficiency for the uptake of the precursor in these different celltypes. Furthermore, the significance of the *de novo* pathway relative to the salvage pathways for synthesis of uridine nucleotides, in combination with compartmentation of the nucleotide pools within the cell nucleus, may lead to diverging utilization of a precursor in different species. During tumour growth there is a low utilization of orotic acid, which seem to be a property characteristic for but not entirely unique for neoplastic growth. In normal cells such as lymphocytes of the spleen the incorporation of orotic acid is also low (Forsdyke, 1968).

This investigation revealed clear differences between mouse and rat liver regarding RNA polymerase activities during regeneration, which reflect different ways of providing for the greater RNA content needed during normal growth. The pattern of RNA polymerase activities found in some hepatomas, i.e. increased activity of RNA polymerase I but not of polymerase II (Duceman & Jacob, 1980) is not restricted to neoplastic growth. A proliferating tissue such as mouse liver after partial hepatectomy revealed the same pattern. The cellular mechanisms related to transcription may thus vary between different growing tissues, but the fundamental characteristics presented in this investigation are not entirely restricted either to normal or to neoplastic growth.

MODULATION OF RNA PRECURSOR METABOLISM DURING HEPATOMA GROWTH.

MODULATION OF 5-FLUOROURACIL METABOLISM BY THYMIDINE.

Mechanisms of action of 5-fluorouracil (5-FU)

In normal rat liver orotic acid is effectively used as a precursor for pyrimidine anabolism and subsequent incorporation into RNA (see page 12), whereas uracil is not readily utilized by the liver cells. It was shown by Rutman, however, that uracil was an efficient precursor for nucleic acids in rat hepatoma (Rutman *et al.*, 1954). These findings led to the investigation by Heidelberger of the utilization of uracil in different tumours compared to the utilization in liver and intestinal mucosa. He showed that all tumours utilized uracil better than orotic acid for RNA and DNA synthesis and that uracil was more efficiently utilized for incorporation into nucleic acids by the tumours than by normal tissues (Heidelberger *et al.*, 1957; Heidelberger, 1981). The wish was therefore to develop a substance resembling uracil which would specifically be taken up by tumours, and thus 5-fluorouracil (5-FU) was synthesized. This substance has been in clinical use since 1957 and is preferentially used against some gastrointestinal carcinomas (Heidelberger, 1974).

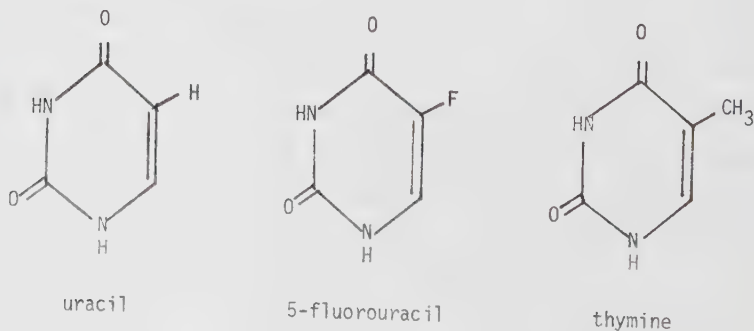


Figure 2. Structure of the pyrimidines uracil, 5-fluorouracil and thymine.

There are two major mechanisms by which 5-FU exerts its antiproliferative effect. One mode of action involves the inhibition of thymidylate synthetase. The synthesis of thymine is made by methylation in position 5' of the uracil ring (Fig. 2). Along the anabolic pathway 5-FU is converted to FdUMP (Fig. 3), which binds to the enzyme thymidylate synthetase and inhibits the reaction dUMP $\longrightarrow$ dTMP (Hartmann & Heidelberger, 1961) (Fig. 3). As a consequence the cellular supply of thymidine nucleotides is depleted and DNA synthesis is inhibited. The other mechanism of action for 5-FU is due to the anabolic conversion to FUTP (Fig. 3), which then becomes incorporated into RNA (Chaudhuri, 1958a). The incorporation into RNA leads to inhibition of the maturation of ribosomal RNA (Stenram & Willén, 1970; Wilkinson *et al.*, 1975). Furthermore, the incorporation into RNA results in impaired methylation of low molecular weight RNA (Glazer & Hartman, 1980) and decreased transcription rate of RNA (Eriksson & Stenram, 1983), and incorporation into mRNA may result in miscoding during translation (Glazer & Hartman, 1981). The RNA-directed cytotoxicity of 5-FU should mainly be related to the impaired processing of rRNA. The relative roles of the DNA-directed and the RNA-directed effects of the drug have been shown to vary, depending on the biological system being studied and the experimental conditions being used. Either or both of these determinants may be of critical importance for the inhibitory actions of 5-FU on tumour proliferation and for the sensitivity of normal tissues to the drug (Mandel, 1981).

Thymidine as a metabolic modulator of 5-FU

When thymidine (TdR) is used in combination with 5-FU a reversal of the cytotoxicity of 5-FU has been found in some cultured tumour cell-lines (Umeda & Heidelberger, 1968; Madoc-Jones & Bruce, 1968). Thus, the

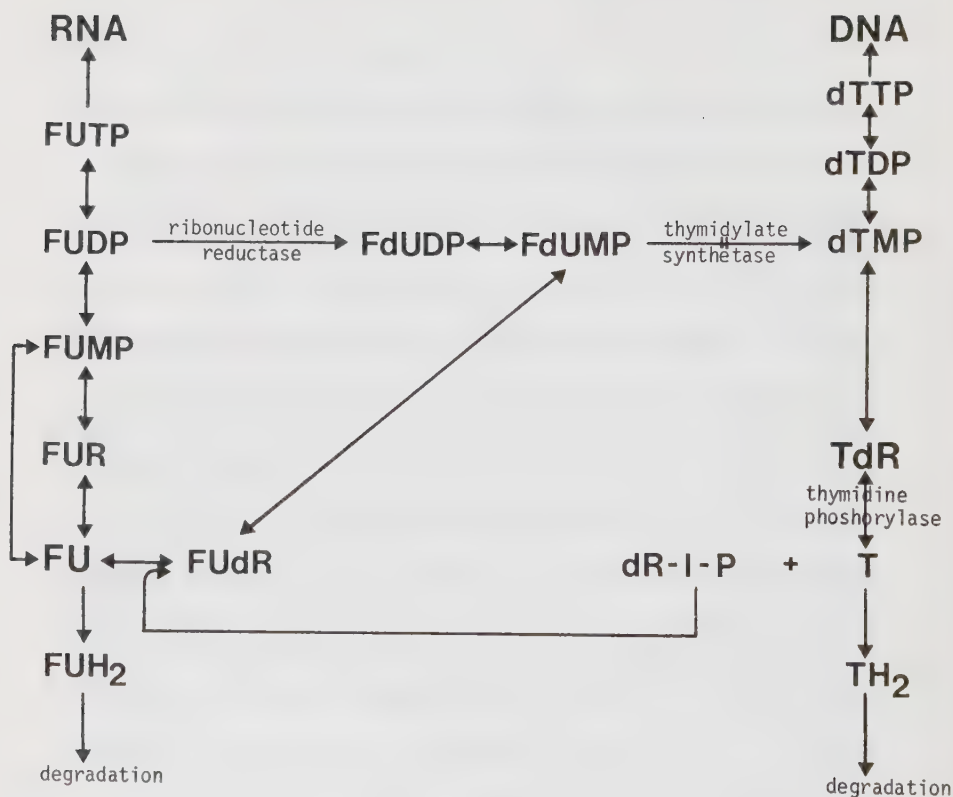


Figure 3.

Metabolism of 5-fluorouracil (5-FU) and thymidine (TdR).

(FU) fluoro-uracil; (FUR) fluoro-uridine; (FUMP, FUDP and FUTP) are the 5'-mono-, di- and triphosphates of uridine, respectively; (FdUDP) fluoro-deoxyuridine-diphosphate; (FdUMP) fluoro-deoxyuridine-monophosphate; (FUdR) fluorodeoxyuridine; (FUH₂) fluoro-dihydrouracil.

(TdR)thymidine; (dTMP, dTDP and dTTP) are the 5'-mono-, di- and triphosphates of thymidine, respectively; (T) thymine; (dR-1-P) deoxyribose-1-phosphate; (TH₂) dihydrothymine.

addition of TdR made the cells overcome the depletion of the dTTP-pool caused by the inhibition of thymidylate synthetase of FdUMP. In other experimental tumours both *in vivo* and *in vitro*, however, the cytotoxicity of 5-FU was increased when it was given in combination with TdR (Santelli & Valeriote, 1978; Spiegelman *et al.*, 1980a; Fried *et al.*, 1981).

The inhibition of DNA synthesis has been demonstrated to occur in two stages (Spiegelman *et al.*, 1980a). For the first 12 h after administration of 5-FU, the effect on DNA synthesis is mediated by the inhibition of thymidylate synthetase. This effect is reversed by additional administration of TdR. During the second phase, approximately 12 h to 96 h after administration, the inhibition of DNA synthesis is no longer reversed by TdR. The results indicate that there is a secondary phase of inhibition of DNA synthesis which is not caused by inhibition of thymidylate synthetase. The secondary phase of action of 5-FU might be due to increased amounts of abnormal RNA (Spiegelman *et al.*, 1980a).

The increased cytotoxicity of 5-FU, when administered in combination with TdR, is accompanied by an augmented incorporation of 5-FU into RNA (Spiegelman *et al.*, 1980a; Young *et al.*, 1981). There are several mechanisms by which TdR may enhance the incorporation of 5-FU into RNA. The increased dTTP-pool may exert feedback regulation on the enzyme ribonucleotide reductase (Martin *et al.*, 1980). This regulation results in an inhibited reduction of 5-FUDP to 5-FdUDP and should lead to increased formation of 5-FUTP and thus to increased incorporation into RNA. Moreover, after conversion of TdR to deoxyribose-1-phosphate and thymine by the enzyme thymidine phosphorylase, thymine may compete with 5-FU for the enzymes of the uracil degradative pathway (Chaudhuri *et al.*, 1958b; Santelli & Valeriote, 1978). This mechanism leads to an increased cellular exposure to 5-FU.

Modulation with thymidine resulting in increased antiproliferative effect of 5-FU on hepatoma AH 130 *in vivo*.

In Paper V the cytotoxic effects of 5-FU were examined after modulation of the incorporation of the drug into RNA by administration of TdR prior to the injection of 5-FU. Treatment with 5-FU alone had an antiproliferative effect on hepatoma AH 130. The antiproliferative effect of 5-FU on hepatoma AH 130 was increased, when the substance was given in combination with thymidine.

The enhanced cytotoxic effect of 5-FU on tumour cells *in vivo* was accompanied by an increased incorporation of the substance into RNA. This augmented incorporation might depend on local effects of TdR on the metabolism of the tumour cells or be due to effects on the liver enzymes for degradation of pyrimidines. The liver is the main pyrimidine catabolizing organ in the body. If the increased incorporation of 5-FU into tumour RNA was a result of a local effect of TdR in the tumour cells, the main results should be reproducible *in vitro*.

Metabolism of 5-FU in AH 130 cells and hepatocytes *in vitro*

Cells of hepatoma AH 130 were incubated *in vitro* with radioactively labelled 5-FU, and the influence of TdR on the metabolism of 5-FU was examined. In this experimental system no increased incorporation of 5-FU into hepatoma RNA could be demonstrated when the cells were pretreated with TdR. The effect of TdR *in vivo* was thus not due to a local effect on the metabolism of the tumour cells. The increased incorporation of 5-FU into hepatoma RNA *in vivo* was probably an effect of decreased catabolism of 5-FU in the liver after competition with thymine for the degrading enzymes. It was thus of interest to examine the effect of TdR on the metabolism of 5-FU in hepatocytes *in vitro*.

There was an extensive catabolism of 5-FU in hepatocytes *in vitro*. This intense catabolism was drastically reduced when the cells were incubated with TdR prior to the addition of 5-FU in analogy with experiments employing liver slices and liver homogenates (Ikenaka *et al.*, 1979; Fujii *et al.*, 1980). The decreased catabolism of the hepatocytes was accompanied by increased amounts of unmetabolized 5-FU in the medium, and these results *in vitro* are in agreement with the findings *in vivo* that TdR leads to increased plasma half-life of 5-FU (Kirkwood *et al.*, 1980; Woodcock *et al.*, 1980; Au *et al.*, 1982) and to reduced amounts of catabolic products in serum (Lee *et al.*, 1979). Thus, concurrent administration of TdR leads to a greater bioavailability of 5-FU.

The striking difference in pyrimidine catabolic capacity between hepatocytes and hepatoma cells was clearly demonstrated in Paper V. In hepatocytes the catabolism of 5-FU was great, whereas in the hepatoma cell medium no radioactivity in the catabolic product α -F- β -alanine could be detected. A low activity of catabolic enzymes is characteristic of tumour cells, with the exception of well differentiated hepatomas (Queener *et al.*, 1971), and it has previously been shown in this laboratory that AH 130 has a low capability of catabolizing thymidine (Engelbrecht *et al.*).

Decreased incorporation of 5-FU into liver RNA after coadministration with thymidine.

In the liver cells both *in vivo* and *in vitro* the coadministration of TdR with 5-FU resulted in a decreased incorporation of 5-FU into RNA. This might depend on reduced uptake of 5-FU or be due to changes in the metabolism of 5-FU in the liver cells. There is often difficulty in discriminating between a change in the membrane transport of a substance and a change in some subsequent intracellular events (Goldman *et al.*, 1981).

Treatment with TdR probably leads to competition for catabolic enzymes for the degradation of 5-FU (Ikenaka *et al.*, 1979; Fujii *et al.*, 1980). The inhibited degradation may lead to expansion of the uracil and thymine pools, which should affect the transport of 5-FU into the cells. Furthermore, 5-FU would be more diluted in the precursor pools after treatment with TdR and thus the incorporation into RNA would be reduced. This explanation applies to the results from liver cells both *in vitro* and *in vivo*.

Modulated host toxicity of 5-FU when given in combination with thymidine.

The combination of TdR and 5-FU markedly increased the effects of 5-FU *in vivo*. If a greater therapeutic response is to be obtained with 5-FU in combination with TdR, compared to 5-FU alone, the increased tumour toxicity must not be accompanied by a corresponding increase in host toxicity. Thus, the increased availability of 5-FU after coadministration of TdR must not merely mimic a higher dose of 5-FU alone. The decrease in body weight of the animals in this investigation was more accentuated when 5-FU was given in combination with TdR than when 5-FU was given alone. TdR thus potentiated the host toxicity of 5-FU. However, there was a differential modulation of the toxicity in different organs as judged by the changes of the incorporation of 5-FU into RNA of different organs. In bone marrow, spleen and kidney the incorporation into RNA was increased by TdR, whereas in liver and intestinal mucosa the incorporation into RNA was decreased. If the toxicity is dependent on RNA-directed actions of 5-FU the changes in incorporation into RNA should express increased toxicity in bone marrow, spleen and kidney while in liver and intestinal mucosa the influence should be diminished. From clinical trials with 5-FU it is reported that TdR potentiates the myelosuppressive effect of 5-FU (Vogel *et al.*, 1979; Kirkwood *et al.*, 1980) whereas gastrointestinal toxicity

is reported not to be increased (Young *et al.*, 1981; Vogel *et al.*, 1979). The toxicity may thus be related to RNA-directed actions of 5-FU. However, the inhibition of DNA synthesis through interference with thymidylate synthetase is undoubtedly an important mechanism for the toxicity of 5-FU. Moreover, TdR is converted to thymine and deoxyribose-1-P by thymidine phosphorylase (Fig. 3), and the deoxyribose is utilized in the conversion of 5-FU to 5-FUdR (Au *et al.*, 1982). Increased levels of circulating 5-FUdR have been reported in patients given 5-FU in combination with TdR (Woodcock *et al.*, 1980; Au *et al.*, 1982). *In vitro* observations on the effects of FUdR have shown that this substance is more toxic than 5-FU (Ardalan & Glazer, 1981). Increased plasma levels of FUdR may thus be of importance for the changes in toxicity of 5-FU after modulation with TdR.

There are thus several mechanisms by which TdR may modulate the *in vivo* effects of 5-FU. In some rodent tumour systems TdR is reported to increase the therapeutic index of 5-FU (Santelli & Valeriote, 1978; Spiegelman *et al.*, 1980b), and concomitantly the incorporation into RNA is increased. However, in clinical trials with 5-FU and TdR in combination the therapeutic index of 5-FU is not increased (Vogel *et al.*, 1979; Kirkwood *et al.*, 1980), but the pharmacokinetic characteristics of 5-FU are changed. The balance of the RNA-directed and the DNA-directed effects of 5-FU is changed in several organs, when 5-FU is given in combination with TdR. The fact that the intestinal toxicity of 5-FU is not potentiated may provide a basis for further development of combination chemotherapy leading to an improved therapeutic index.

SUMMARY

RNA synthesis in regenerating mouse liver, at 24 h and 48 h after partial hepatectomy, was studied by incorporation of [methyl- ^{14}C]-methionine and [^{14}C]orotic acid *in vivo* and by determination of RNA polymerase activities in liver nuclei *in vitro*. Some characteristics related to the synthesis of RNA during normal and neoplastic growth are discussed.

1. Incorporation of [methyl- ^{14}C]methionine and RNA polymerase activities indicated an increased synthesis of ribosomal RNA at 24 h and 48 h, whereas a decreased synthesis of hn RNA was observed at these times after partial hepatectomy.
2. Total transcription was increased by 32% at 24 h. Thus, the increase in transcriptional activity is not as accentuated in regenerating mouse liver as in regenerating rat liver.
3. There were no significant changes in free RNA polymerase activities at 24 h and 48 h after partial hepatectomy.
4. Anaesthesia and/or surgery influenced transcriptional activity. At 24 h after operation total transcription was depressed in sham-operated animals compared to unoperated controls. At 48 h the transcriptional activity was completely restored to the levels revealed by the unoperated controls. An effect of the operation procedure was also found on the incorporation of orotic acid. At 24 h the incorporation of the precursor into the acid soluble fraction and into RNA was decreased in sham-operated controls compared to unoperated controls.

5. Incorporation of [^{14}C]orotic acid resulted in an increased specific radioactivity of total RNA of 59% at 24 h, when a labelling time of 120 min was used. At 48 h, however, no increased specific radioactivity of RNA could be demonstrated.
6. There was a difference in the utilization of orotic acid and uridine between normal, resting liver and hepatoma AH 130. Orotic acid was effectively utilized for RNA synthesis in normal liver but not in hepatoma. Uridine was efficiently incorporated into hepatoma RNA but not into RNA of normal liver.

The modulating effect of thymidine on the metabolism and toxicity of 5-FU was studied *in vivo* and *in vitro* in rat liver and hepatoma.

7. Thymidine potentiated the antitumour effect of 5-FU on hepatoma AH 130, and the enhanced cytotoxic effect of 5-FU on tumour cells *in vivo* was accompanied by an increased incorporation of the substance into tumour RNA.
8. There was a striking difference in pyrimidine catabolic capacity between hepatocytes and hepatoma cells *in vitro*. In hepatocytes the catabolism of 5-FU was great, whereas in hepatoma cells no catabolism of 5-FU could be demonstrated.
9. The enhanced incorporation of 5-FU into tumour RNA *in vivo* after pretreatment with thymidine was not related to local effects on the tumour cells but rather to an increased bioavailability of the drug.
10. In liver cells both *in vivo* and *in vitro* coadministration of thymidine with 5-FU resulted in a decreased incorporation of 5-FU into RNA.

11. Coadministration of thymidine *in vivo* resulted in increased but also modulated host toxicity of 5-FU. In bone marrow, spleen and kidney the incorporation into RNA was increased, whereas in liver and intestinal mucosa the incorporation into RNA was decreased.

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Incorporation of Orotic Acid into Nucleotides and RNA in Mouse Organs during 60 Minutes

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Summary: Mouse kidney and liver were found to increase their levels of radioactivity above that of serum from 2 to 60 min after administration of [6-¹⁴C]orotic acid. In spleen, thymus and brain, the radioactivity level reached a maximum soon after the injection and then decreased, as did that in serum. Sixty minutes after the injection, 44% of the administered isotope dose was found in the kidneys, 22% in the liver and 0.75% in the spleen. The ¹⁴C activity in liver UTP increased rapidly and then remained con-

stant for 60 min. The ratio between the activities in uridine phosphates and UDP-sugars was 3:4 from 10 - 60 min after injection. In the liver and kidneys, the RNA ¹⁴C activities at 60 min after injection were 15% of the activity in their acid-soluble fractions. Intraperitoneal administration was found to be preferable to intravenous administration for studies on nucleotides and RNA in mouse liver, due to the delayed incorporation of the [¹⁴C]orotic acid activity into the nucleotide pool.

Einbau von Orotsäure in Nucleotide und RNA von Mäuseorganen innerhalb von 60 Minuten

Zusammenfassung: In den Nieren und in der Leber von Mäusen erhöhten sich die Niveaus der Radioaktivität über das in Serum 2 bis 60 Minuten nach Injektion von [6-¹⁴C]Orotsäure. In Milz, Thymus und Gehirn wurden die höchsten Aktivitätsniveaus gleich nach der Injektion registriert und nahmen genau wie die Serumaktivität ab. Sechzig Minuten nach der Injektion wurden 44% der injizierten Aktivität in den Nieren, 22% in der Leber und 0.75% in der Milz wiedergefunden.

Die ¹⁴C-Aktivität der Leber UTP stieg schnell an und blieb während 60 Minuten konstant. Das

Verhältnis zwischen den Uridinphosphataktivitäten und den UDP-Zuckeraktivitäten war etwa 3:4 von 10 bis 60 Minuten nach der Injektion. In der Leber und den Nieren betrugen die RNA-¹⁴C-Aktivitäten, 60 Minuten nach der Injektion, 15% der Aktivität ihrer säuerlöslichen Fraktionen. Bei Analysen von Nucleotiden und RNA in der Leber ist ein intraperitoneales Injektionsverfahren gut geeignet, da es eine verzögerte Inkorporierung der injizierten Orotsäure in den Nucleotidpool gibt.

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Abbreviations: i.p. = intraperitoneal; i.v. = intravenous.

Studies on nucleic acid synthetic activity by use of labelled precursors require a thorough insight into the changes occurring in the nucleotide pool during the pulse period^[1-5]. The specific activity in the pool of UTP, one of the immediate RNA precursors, has been analyzed in different living materials after the addition of [³H]uridine or [¹⁴C]orotic acid^[3,6]. Models based on the specific activity in the immediate precursor pool have been suggested for the calculation of the amount of RNA synthesized in lymphocytes^[2], in mouse embryos^[7] and in rat liver^[8].

For analysis of RNA synthesis in the mammalian liver, orotic acid is considered to be a favorable precursor substance because of its good incorporation into RNA^[9-16].

Orotic acid is incorporated into nucleic acids via the pathway of de novo synthesis which involves the stepwise formation of nucleotides from relatively small molecules. In order to study RNA synthesis in mouse organs, especially the liver, we analyzed the incorporation of [¹⁴C]orotic acid into nucleotides and RNA in liver, kidney, spleen, thymus and brain at 2, 5, 10, 20 and 60 min after the isotope injection. The acid-soluble material from liver was isolated and fractionated into orotic acid, uridine, UTP, UDP, UMP and UDP-sugars.

Techniques for intravenous injection into the tail vein of mouse and rat have been described^[8,17]. In the mouse, intravenous administration of the orotic acid was difficult to accomplish, and the small body volume did not allow flushing of the canula and vein with 0.9% saline. Therefore we compared the results obtained after tail vein injection with those obtained after intraperitoneal injection.

Materials and Methods

Animals. Fifty young male NMRI-mice weighing 26-28 g (Anticimex, Uppsala, Sweden) were used for the experiments. They were kept under constant conditions regarding light and temperature and had free access to standard food and drinking water. All experiments were carried out between 8 and 12 a.m.

Sacrifice of animals and preparation of tissues. The animals were killed at 2, 5, 10, 20 or 60 min after the injection of 1.75 μ Ci [⁶⁻¹⁴]orotic acid, spec. act. 61 mCi/mmol (Amersham). The orotic acid was dissol-

ved in 0.9% NaCl and administered in 200 μ l intraperitoneally or in the tail in 75 μ l.

Ninety seconds prior to death the mice were anaesthetized in an oxygen/ether atmosphere. The liver was rapidly pulled out, squashed between precooled aluminium blocks and immersed in liquid nitrogen. Blood was taken from the peritoneum, coagulated, and the serum was collected and frozen until analysis. The spleen, one kidney, the thymus and the cerebrum were rapidly dissected out and frozen in liquid nitrogen.

The possible influence of intraperitoneally injected isotope on intraperitoneally sampled blood serum was examined. In 14 animals, killed 5 minutes after tail vein or intraperitoneal injection, blood was collected from the brachial vessels according to Young and Chambers^[18].

Tails were hydrolyzed in 3M KOH for 6 h at 70 °C. The supernatants were acidified with HClO₄ and used for calculation of radioactivity.

Quantitative analysis of nucleic acids. Frozen thymus (50-100 mg), spleen (100 mg), kidney (100 mg), brain (200 mg), or liver (200 mg) tissue was weighed and homogenized in 2 ml of distilled water. Two 1-ml samples were removed from the homogenate. One sample was analyzed immediately while the other was frozen in liquid nitrogen until use. The samples were prepared according to the Schmidt and Tannhauser method, modified by Munro and Fleck^[19] as described previously^[20].

Incorporation of orotic acid. The acid-soluble fraction, the RNA hydrolysate and the DNA-hydrolysate were used for estimation of the incorporation of orotic acid. The radioactivity of samples in 0.2M HClO₄ from the different organs, from serum and from hydrolyzed tails was counted in a Packard Scintillator.

Analysis of the liver nucleotide fraction. Fractionation of the liver acid-soluble material was carried out essentially according to Lust^[21] and Raaen^[22]. The liver was weighed, pulverized under liquid nitrogen and dispensed with mechanical stirring into ice-cold 0.4M HClO₄, 7 ml/g. The mixture was homogenized at 0 °C, and after centrifugation for 15 min at 0 °C, the supernatant was adjusted to pH 7.0 with ice-cold 7M KOH. The precipitated KClO₄ was sedimented by centrifugation. The supernatant was lyophilized and then suspended in 1N CH₃COOH and clarified by centrifugation. Portions of the supernatant were applied onto a polyethyleneimine-impregnated aluminium thin-layer plate and chromatographed in two dimensions. The radioactively labelled orotic acid, uridine, UTP, UDP, UMP, UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine and UDP-glucuronic acid, identified by the corresponding marker compounds under ultraviolet light, were cut out and eluted from the PEI-cellulose layer and the radioactivity was counted in a Packard Scintillator.

Results and Discussion

The serum radioactivity 5 min after the injection of [^{14}C]orotic acid was less than 5% of the value calculated on the assumption that all the injected isotope remained in the blood serum, Table 1. The 60-min values were about 10% of those 2 min after injection, Fig. 1A. This is in agreement with values reported from analyses in the rat^[10]. Thus, a rapid penetration of the orotic acid into the tissues must have occurred, and this was confirmed by analysis of the acid-soluble fractions from different organs, especially the kidneys and the liver, Fig. 1B. The time course of the activity in the acid-soluble fraction indicates that kidneys and liver accumulated radioactivity to a level above that of serum.

Sixty minutes after the injection, the acid-soluble fraction of the liver (mean weight 2.07 g) contained 22% of the total administered isotope dose, that of the kidneys (mean weight 0.45 g) 44%, and that of the spleen (mean weight 0.17 g) 0.75%. Similar results have been obtained in the rat^[13,24]. The serum isotope concentrations were higher after intraperitoneal injection than after intravenous injection, Fig. 1A. This difference was reduced when blood was sampled from the brachial vessels, indicating that the

isotope administered intraperitoneally contaminated the blood sampled, from the peritoneum, Table 1^[25]. The high level of orotic acid radioactivity in the liver samples at 2, 5 and 10 min after intraperitoneal injection also resulted from unabsorbed orotic acid, Fig. 2A. A delayed distribution of the precursor after intraperitoneal injection, as compared to intravenous injection, was supported by the isotope incorporation into the liver nucleotides, as it in general showed a slower increase during the first few minutes following intraperitoneal injection, Fig. 2A.

A rapid conversion of orotic acid into uridine nucleotides is known to occur in rat liver^[13,26] and was also found in mouse liver, Fig. 2A,B. The total radioactivity of the uridine phosphates was about 30%, that of the UDP-sugars about 42% and that of uridine about 10% of the total activity in the acid-soluble fraction 10 - 60 min after intraperitoneal or intravenous injection. The high radioactivity in the UDP-sugars as compared to the uridine phosphates was consistent with results reported by other authors^[6,27], but the percentage amount of uridine activity was higher than that in the rat liver^[13]. Unidentified catabolites, not separated quantitatively from uridine by the chromatographic method employed, may have contributed to this anomaly.

Table 1. Radioactivity in serum and tails.

Mean $\pm$ S.D. Blood collected from the brachial vessels.

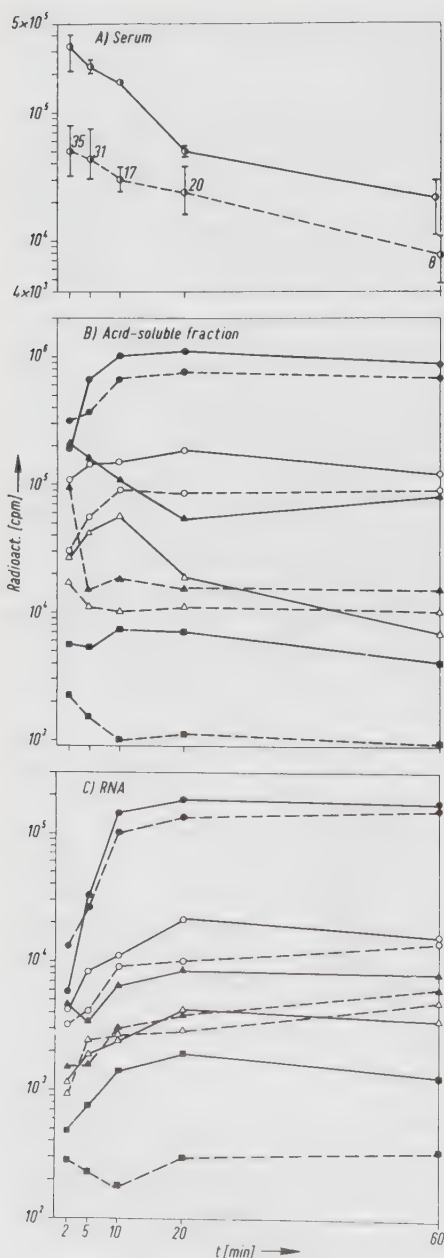
Injection technique	No. of mice	Serum volume calculated ^{a)} [ml]	Counts per minute			
			Total dose	per ml serum		in tails % of total at 5 min
				theoretical at injection ^{b)}	measured at 5 min	
i. v.	7	1.54 $\pm$ 0.04	900000	585000	11738 $\pm$ 4110	24% $\pm$ 25
i. p.	7	1.56 $\pm$ 0.06	900000	577000	13865 $\pm$ 1924	0.66% $\pm$ 0.34

a) 5.6 ml/100 g body weight^[23]. b) Total dose/serum volume.

Table 2. Nucleic acids.

Mean $\pm$ S. D.

	No. of mice	Liver	Kidneys	Spleen	Thymus	Brain
RNA [mg/g]	35	7.38 $\pm$ 1.74	4.69 $\pm$ 0.51	7.14 $\pm$ 1.18	6.27 $\pm$ 0.79	1.90 $\pm$ 0.20
DNA [mg/g]	35	2.59 $\pm$ 0.58	4.56 $\pm$ 1.10	20.96 $\pm$ 5.01	37.28 $\pm$ 7.77	1.76 $\pm$ 0.05



Ten to sixty minutes after the isotope injection, liver and kidney RNA radioactivity was around 15% of the total activity in the acid-soluble fraction of the respective organs. This is in accordance with results from rat liver, as reported by Yu and Feigelson^[28], who found the RNA activity to be 14% of the total activity 20 min after injection of [¹⁴C]orotic acid. In spleen, thymus and brain, we found the activity in RNA at 60 min to be more than 15%, in some cases almost 100% of the activity in the acid-soluble fraction, Fig. 1B,C. Different time courses for the incorporation and differences in turnover of the injected precursor could explain the dissimilarities. The high level of activity in the kidney RNA could be explained by the fact that labelled metabolites formed in the liver from orotic acid were used as nucleotide precursors by the kidney, Fig. 1C. In the kidney acid-soluble fraction, [¹⁴C]orotic acid excreted unchanged in the urine probably further raised the level of radioactivity, Fig. 1B^[24].

A relevant comparison of the RNA synthetic activity in the different organs should include analysis of the specific activities in the UTP-pools during the pulse period. A slow, constant uptake of orotic acid into the liver UTP-pool is favorable for studies on RNA synthesis^[8], and should be improved by intraperitoneal administration.

We would like to thank Mrs. Eva Dahlberg for skilled technical assistance.

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Fig. 1. Radioactivity in serum, acid-soluble material and RNA after intraperitoneal (—) or intravenous (---) injection of [⁶⁻¹⁴C]orotic acid.

The numbers beside the points on the serum graph denote the cpm, as a percentage of the total administered cpm, detectable in tails from the mice injected in the tail vein at each time. Each point represents the mean from 2-4 mice. Bars represent the difference between the highest and the lowest serum value at each time. ○, Serum; ●, kidney; ○, liver; ▲, spleen; △, thymus; brain.

Abscissa: minutes after injection; Ordinate: Radioactivity per ml or g.

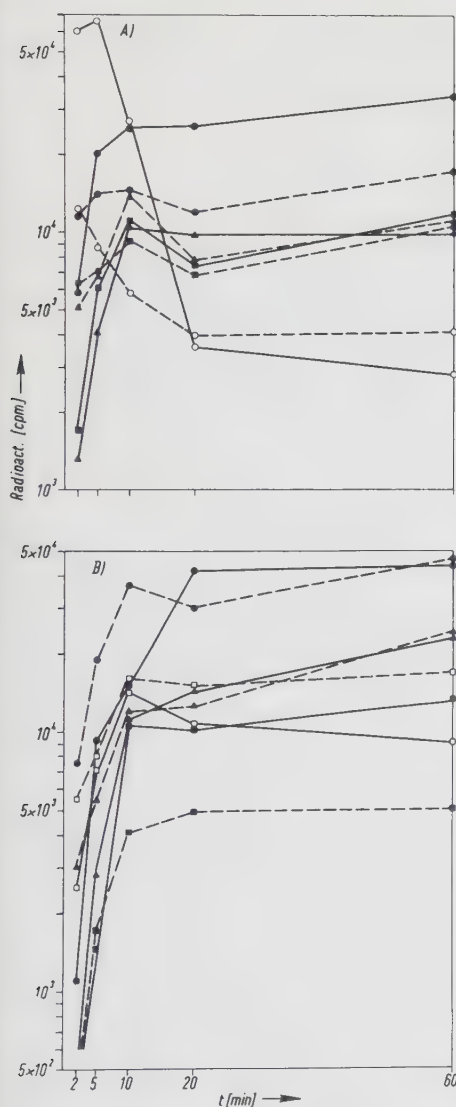


Fig. 2. Radioactivity in orotic acid, uridine and uridine nucleotides in the liver after intraperitoneal (—) or intravenous (---) injection of $[6-^{14}\text{C}]$ orotic acid.

A) $\circ$, Orotic acid; $\bullet$, UTP; $\blacksquare$, UDP; $\blacktriangle$, UMP.

B) $\bullet$, UDP-glucose + UDP-galactose; $\blacktriangle$, UDP-N-acetylglucosamine; $\blacksquare$, UDP-glucuronic acid; $\circ$, uridine.

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METABOLISM OF [5-³H]URIDINE IN MOUSE AND SPECIFICITY FOR LABELING OF LIVER RIBONUCLEIC ACID*

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Abstract—1. The amount of [³H]uridine incorporated into the acid soluble fractions of different mouse organs decreased in the following order: liver > kidney > spleen > thymus > brain.

2. Ten minutes after injection, radioactively labeled uracil, dihydrouracil, β -UIP and β -alanine together amounted to 17% of the liver acid soluble radioactivity and 7% of that in blood, whereas ³H₂O made up 5% and 45%, respectively.

3. At 10 minutes after injection the radioactivity of the uracil nucleotides and UDP-sugars together amounted to 35% of the total liver acid soluble radioactivity.

4. The mouse liver seems to utilize [³H]uridine and [¹⁴C]orotate to a similar extent, but [³H]uridine was more effectively incorporated into UTP and RNA of mouse liver than of rat liver.

INTRODUCTION

Cellular metabolites for RNA synthesis are anabolized along two main pathways: the *de novo* synthetic pathway and the so-called salvage pathway. Calculation of RNA synthetic activity in different biological materials is based on the analysis of incorporation of various labeled precursors into the RNA. Precursors in common use for such investigations are orotic acid—incorporated along the *de novo* pathway—and uridine—incorporated along the salvage pathway. RNA and nucleotide metabolism have been studied by labeled uridine in a wide variety of biological materials. Labeled orotic acid has also often been used, and since the careful analysis of orotic acid metabolism by Hurlbert and Potter (1954), this precursor has been by far the most frequently used radionuclide for studies of liver RNA synthesis. Contradictory results about the *in vitro* incorporation of uridine and orotic acid into liver cells and liver slices have been reported (Schreiber *et al.*, 1974; Hogans *et al.*, 1971; Tseng & Gurpide, 1973). However, orotic acid is considered to be a better precursor than uridine for *in vivo* studies of RNA synthesis in mammalian liver (Yu & Feigelson, 1970; Witschi, 1972; Ord & Stocken, 1972). Because the turn-over rate for tritium activity from [³H]orotic acid is lower than from [³H]uridine in liver, orotic acid was expected to be more effective for long-term incorporation studies (Yu & Feigelson, 1970). Furthermore, results from a recent study suggest that rat liver RNA is more specifically labeled with orotic acid than with uridine (Dahnke, 1975a).

Differences in metabolism and catabolism of administered RNA precursors in different organisms, in different tissues in the same organism, and in the

same organism under different physiological conditions can lead to inconsistent results concerning RNA synthetic activity by using alternative radioactive precursors. Inconsistent results can also be obtained by using different methods for analysing RNA radioactivity. Information about the overall metabolism of the precursors and knowledge of the specific radioactivity of the immediate nucleoside triphosphate pools, e.g. UTP and CTP, are essential for calculating the rate of RNA synthesis from the incorporation of labeled precursors (Bucher & Swaffield, 1969; Vacha & Seifert, 1975; Cooper, 1972; Goody & Ellem, 1975). The present study evaluated the distribution of radioactivity from [³H]uridine in the mouse by analysis of the acid soluble fractions of blood, liver, kidney, spleen, thymus and cerebrum. As the circulating amount of an administered precursor is largely limited by the catabolism that occurs mainly in the liver, we analysed the availability and catabolism of [³H]uridine in blood and liver and the incorporation of radioactivity into liver anabolic products at intervals during 60 min after injection. We also compared two different methods for analysis of liver RNA radioactivity, as unspecific label from the administered [³H]uridine has been found to interfere with measurements of the specific activity of rat liver RNA (Dahnke, 1975a). Certain results obtained in this study after injection of [³H]uridine are discussed in relation to results from a previous investigation of the incorporation of radioactivity from [¹⁴C]orotic acid into mouse organs (Lewan *et al.*, 1975).

MATERIALS AND METHODS

Animals

Young male NMRI mice weighing 26–28 g (SPF quality, Anticimex, Sweden) were used. The animals were kept under constant light and temperature conditions and had free access to standard food and drinking water. All experiments were carried out between 8 a.m. and noon

* anabolism

*Supported by grants from the Carl Trygger Foundation, the Foundation of Director Albert Pahlsson and the Royal Physiographical Society.

Radionuclide and measurement of radioactivity

[5-³H]Uridine, spec. act. 5000 mCi/m-mole (Amersham, U.K.). All radioactivity measurements were performed with a Tricarb liquid scintillation spectrometer (Packard Inst., Co.).

Tissue sampling

The animals were killed 2, 5, 10, 20, or 60 min after an intraperitoneal injection of 75 μ Ci [³H]uridine in 75 μ l 0.9% NaCl. At quantitative analysis of cellular metabolites, inactivation of enzymatic activity is required immediately on tissue sampling (Tseng *et al.*, 1971). The liver nucleotide concentration is extremely sensitive to hypoxia, stress, and narcosis (Faupel *et al.*, 1972). To reduce the effect of these factors, special precautions must be taken. The animals were anaesthetized in an ether-oxygen atmosphere (Buchner & Swaffield, 1966) 90 sec before tissue sampling. The livers were rapidly extracted, squashed between pre-cooled aluminium-blocks, and immediately immersed in liquid nitrogen. To further minimize the hypoxic effect on the liver cells, blood was sampled from the peritoneal cavity after liver resection. After blood collection, mouse tissues were extracted and frozen in the following order: one kidney, the spleen, the thymus, and the cerebrum. In one series of experiments, blood was sampled from the brachial vessels before liver resection (Young & Chambers, 1973; Yngner *et al.*, 1975); in another series, the intraperitoneally sampled blood was coagulated at room temperature (+20°C) and the serum was collected and frozen until analysis.

Analysis of radioactivity in acid soluble and RNA fraction from different organs

Frozen thymus (50–100 mg), spleen (100 mg), kidney (100 mg), cerebrum (200 mg), and liver (200 mg) were homogenized in distilled water. Two independent samples were removed from each homogenate. They were fractionated and analysed for nucleic acids according to the Schmidt and Tannhauser method as modified by Munro and Fleck (1966). Radioactivity was measured in aliquots from the acid soluble fraction, the RNA fraction, and the DNA fraction.

Preparation of liver RNA

In one series of experiments, RNA was isolated from liver tissue according to the method of Kirby (1965) (using 4-aminosalicylate in the phenol kresol mixture) 20 min after administration of [5-³H]uridine. The specific activity of the RNA was compared with the specific activity of the liver RNA-fraction obtained by the modified Schmidt-Tannhauser method (Munro & Fleck, 1966).

Analysis of radioactivity in uridine and in uridine metabolites in liver and blood acid soluble fractions

The frozen liver was pulverized under liquid nitrogen and dispensed with mechanical stirring into ice cold 0.4 M HClO₄, 7 ml/g. The mixture was homogenized at 0°C. After centrifugation the supernatant was adjusted to pH 7.0 with 7 M KOH. The precipitated KClO₄ was sedimented by centrifugation. The supernatant was lyophilized, taken up in water, and aliquots were applied onto polyethyleneimine (PEI)-cellulose coated aluminium sheets. Blood was deproteinized by the addition of 0.8 M HClO₄, neutralized with 7 M KOH, and the neutral fraction cleared by centrifugation. The separation of nucleotides (UMP, UDP, UTP) and UDP-sugars (UDPG, UDP-Gal, UDPAG, and UDPGA) was performed by two-dimensional thin-layer chromatography (Lust & Sahud, 1972). Uridine, uracil, dihydrouracil, β -alanine, and β -ureidopropionic acid (β -UIP) were separated essentially according to the method described by Raaen and Kraus (1968). The radioactively labeled metabolites were identified by the corresponding unlabeled marker compounds under u.v. light or with

acidified *p*-dimethylaminobenzaldehyde (Fink *et al.*, 1956) and confirmed by autoradiography (X-ray film, Agfa Gevert). The marker compounds were purchased from Sigma Chemical Co., St. Louis, U.S.A. The identified spots were cut out, the metabolites were eluted from the PEI-cellulose, and the radioactivity was assayed by liquid scintillation counting. Volatile radioactivity was calculated as the difference between total radioactivity and non-volatile radioactivity determined in 25 μ l of the neutralized fractions before and after evaporation.

RESULTS

Radioactivity in blood

The total acid soluble radioactivity and the uridine radioactivity registered in blood after administration of [³H]uridine were dependent on the blood sampling technique, i.e. during the first 10–20 min after injection, the level was enhanced in intraperitoneally sampled blood compared with blood sampled at the brachial vessels (Fig. 1). The labeled uridine in blood sampled at the brachial vessels should represent the precursor concentration in contact with the peripheral organs. The level of total acid soluble radioactivity in blood sampled at the brachial vessels amounted to 1–2% of the totally administered radioactivity from 2 min after injection; and at 20 min, only 2% of the acid soluble radioactivity could be attributed to [³H]uridine.

In intraperitoneally sampled blood, the uridine radioactivity decreased rapidly; at the end of the pulse period, the level was reduced to approx 0.01% of the totally administered dose. A high level of volatile radioactivity was obtained already 2 min after injection; at 60 min, the level was 60 times that of uridine, i.e. about 60% of the total acid soluble radioactivity (Table 1). The radioactivity in the uridine catabolic products uracil and dihydrouracil was lower

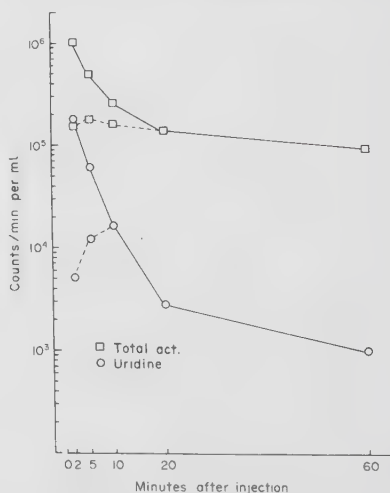


Fig. 1. Radioactivity (counts/min per ml) in blood 2–60 min after injection of 75 μ Ci [³H]uridine. (—) Intraperitoneally sampled blood; (---) blood sampled at the brachial vessels. Each point represents the mean of blood from 3 animals.

Table 1. Distribution of radioactivity in blood and liver acid soluble fractions calculated as counts min $\times 10^{-3}$ ml or gram and in % of total acid soluble radioactivity

Time after injection (min)	Uridine		Uracil + dihydrouracil		β -Alanine + β -UIP ^a		Volatile		Uracil nucleotides		UDP-sugars	
	Blood	Liver	Blood	Liver	Blood	Liver	Blood	Liver	Blood	Liver	Blood	Liver
2	185.8	52.6	24.1	11.3	8.0	49.8	140.3	33.1	—	50.1	—	4.3
	(%) 37.4	9.7	4.9	2.1	1.6	9.2	28.3	6.1	—	9.3	—	0.8
10	17.9	12.0	9.5	7.2	8.9	147.3	114.5	45.2	—	201.6	—	121.0
	(%) 7.0	1.3	3.7	0.8	3.5	16.2	45.0	5.0	—	22.1	—	13.3
60	1.0	3.1	2.2	1.7	1.2	6.4	59.0	57.9	—	99.3	—	87.4
	(%) 1.0	0.8	2.3	0.4	1.7	1.6	61.3	14.1	—	24.1	—	21.2

^a β -Ureidopropionic acid (β -UIP).

The values represent the mean of 3 pooled samples at each interval.

than the uridine radioactivity 2 and 10 min after injection, and approx twice that of uridine at 60 min (Table 1). Labeled β -alanine and β -UIP accumulated in blood for 10 min and then decreased, and at the end of the pulse period, the radioactivity was in the same range as that of uridine.

Coagulation of blood at room temperature drastically increased the amount of labeled uridine catabolic products (Table 2). The results of the enzymatic activity was most pronounced during the first minutes after injection, when a major part of the blood radioactivity still remained as [3 H]uridine.

Radioactivity in the acid soluble fraction of different organs

Figure 2 shows the rate of incorporation of radioactivity into the acid soluble fraction of different organs. In the analysed organs, except cerebrium and thymus, the radioactivity per gram tissue in the acid soluble fractions exceeded the total acid soluble radioactivity per ml blood 5-60 min after injection. Contrary to the conditions in spleen and thymus, liver and kidney accumulated radioactivity until approx 10 min after injection. In cerebrium, radioactivity accumulated continuously during the 60 min pulse period.

In the liver, the uridine radioactivity was less than that of blood sampled in the peritoneal cavity for at least 10 min after injection, but was three times the blood level at 60 min (Table 1). Contrary to blood, liver uracil and dihydrouracil radioactivity was less than the uridine level during the pulse period and never exceeded 60% of the uridine level. At 10 min

after administration, approx 16% of the ~~totally administered radioactivity~~ ^{*} was obtained in the liver as labeled β -alanine and β -UIP, but this value was reduced to 3.8% at 60 min. Tritium radioactivity was rapidly incorporated into liver uracil nucleotides and UDP-sugars, and at 10 min after injection of the [3 H]uridine, the radioactivity of these metabolites together made up about 35% of the acid soluble radioactivity. Thus, among the analysed liver metabolites, these anabolic products contained most of the radioactivity from the injected [3 H]uridine during the period of analysis. Actually, more of the administered radioactivity was identified in nucleotides and UDP-sugars than in the volatile fraction. Volatile radioactivity, most probably 3 H $_2$ O, was formed almost immediately upon administration of [3 H]uridine. An even distribution of 3 H $_2$ O between blood and liver was found at 60 min after administration (Table 1).

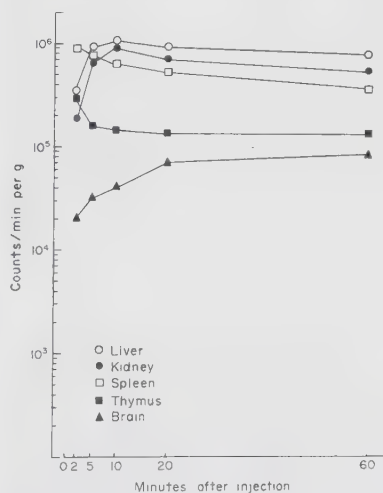


Fig. 2. Radioactivity (counts/min per g wet wt) in acid soluble fraction of mouse organs 2-60 min after injection of [5 - 3 H]uridine. Each point represents the mean of 3 animals.

* total acid soluble fraction

** 1.6%

Table 2. Radioactivity in uracil + dihydrouracil from blood (frozen in liquid nitrogen) and serum (after coagulation at $+20^\circ\text{C}$), calculated as % of the uridine radioactivity

Time after injection (min)	Blood, frozen Uracil + dihydrouracil	Serum, unfrozen Uracil + dihydrouracil
2	13%	717%
10	53%	372%
60	220%	109%

Values are means of 3 pooled samples at each time-point.

Table 3. Radioactivity^a (counts min⁻¹ · 10⁻³ per g wet wt) in acid soluble fractions, UTP, and RNA 20 min after administration of [5-³H]uridine to mouse and rat^b

Animal	Organ	Acid soluble fraction	UTP	RNA Kirby method	RNA Schmidt-Tannhauser method
Mouse	Liver	900 (100%)	90 (10%)	15 (1.7%)	101 (11%)
	Kidney	720 (100%)	—	—	85 (12%)
	Spleen	550 (100%)	—	—	190 (35%)
	Thymus	135 (100%)	—	—	25 (19%)
	Brain	70 (100%)	—	—	2.7 (4%)
Rat	Liver	1080 (100%)	1.3 (0.12%)	0.3 (0.03%)	6.5 (0.6%)

^a Values for radioactivity in acid soluble fractions and Schmidt-Tannhauser RNA fractions represent the mean of 3 animals.

UTP and RNA were isolated from pooled livers from 7 mice or 3 rats.

^b Male Sprague-Dawley rats (160–180 g) were injected intraperitoneally with 300 μ Ci [5-³H]uridine in 300 μ l saline.

The [5-³H]uridine was injected intraperitoneally in 75 μ l (75 μ Ci) to mouse.

Radioactivity in nucleic acid fractions from different organs

The amount of radioactivity obtained in the RNA fraction from the different organs during the alkaline extraction, according to the Schmidt-Tannhauser method, increased rapidly after injection of the labeled uridine, but remained relatively constant from 5–10 min after administration. The largest amount of radioactivity per gram tissue was extracted from the spleen (Table 3). At 20 min, the radioactivity from the spleen amounted to 35% of the total acid soluble radioactivity, whereas the corresponding values for liver, kidney, thymus, and cerebrum were 11, 12, 19, and 4%, respectively. Further radioactivity, i.e. 0.5–4% of the acid soluble radioactivity in the respective organs, was released during the hot acid hydrolysis of DNA.

A comparison was made between the level of radioactivity in the liver RNA fraction obtained according to the Schmidt-Tannhauser method and the level in the isolated RNA obtained according to the Kirby method. The radioactivity in isolated RNA from mouse liver was found to be about 15% of that obtained by alkaline extraction of RNA from PCA extracted liver tissue. A corresponding value for rat liver was about 5%. At 20 min after injection, the radioactivity of mouse liver RNA amounted to 1.7% of that in the acid soluble fraction, whereas the value for rat liver was only about 0.03% (Table 3).

DISCUSSION

The results indicate that radioactivity from the intraperitoneally administered [5-³H]uridine was rapidly taken up from the peritoneal cavity and maximally incorporated into the acid soluble fraction of the different organs within 20 min after injection. A rapid distribution of radioactivity throughout the body should be expected because a high level of volatile radioactivity (i.e. ³H₂O) appeared in the blood almost immediately on administration of [5-³H]uridine. Liver and kidney accumulated radioactivity during 10 min, and increased their levels of radioactivity over that in blood. Similar results were reported for the rat

(Hogans *et al.*, 1971). The high acid soluble radioactivity in spleen at 2 min after injection might indicate a high affinity by this organ for uridine. However, because the spleen, similar to a number of tumours, seems to be without detectable uridine catabolic activity (Cooper *et al.*, 1972), the level of acid soluble radioactivity it demonstrates, should be directly affected by the level of circulating labeled metabolites. The same explanation is perhaps relevant to the acid soluble radioactivity from thymus.

Previous results indicated that intraperitoneally administered [5-³H]uridine was able to cross the blood-brain barrier only after conversion to UMP (Piccolo *et al.*, 1971), and that the blood-brain barrier played an important role in the uptake of RNA-precursors into the brain cells (Jakoubek, 1976). Our results show that only a small part of the administered radioactivity could be recovered from the brain tissue. Investigations performed on rat suggest that, despite an extensive catabolism of the injected uridine occurring in the animal, a sufficient amount escapes the degradative pathways to permit incorporation of radioactivity into both acid soluble fraction and RNA of the brain cells (Hogans *et al.*, 1971).

The high levels of catabolic products in blood and liver acid soluble fractions soon after injection should be correlated to the high uridine phosphorylase activity localized to the blood plasma and liver cell membranes (Tseng *et al.*, 1971; Tseng & Gursipide, 1973; Guroff & Rhoads, 1969). A pronounced uridine catabolic activity was demonstrated in mouse blood at coagulation in room temperature (+20°C) (Table 2). Based on these results and on a similar observation of rapid uridine catabolism in dog plasma (Tseng *et al.*, 1971), it is obvious that non-optimum conditions during blood sampling must be avoided to obtain relevant measurements of metabolite levels in blood.

A certain catabolism of the administered [5-³H]uridine should have occurred in the peritoneal cavity before incorporation into the blood. The high levels of radioactivity associated with uracil and dihydro-uracil in intraperitoneally sampled blood already 2 min after injection, i.e. when the intraperitoneally administered [5-³H]uridine was not yet absorbed from the peri-

toneal cavity, support the idea that uridine was readily converted to uracil by uridine phosphorylase located in the plasma membranes (Tseng & Gurpide, 1973; Dahnke & Mosebach, 1975b). Some of the uracil is apparently released extracellularly, and at intraperitoneal blood sampling it could be recovered in the blood acid soluble fraction. That the further catabolism of the uridine was confined essentially to the liver cells (Reichard & Sköld, 1958) was supported by the high radioactivity recovered in liver β -alanine + β -UIP. Labeled β -alanine accumulated also in rat liver after administration of ^3H -labeled uridine (Dahnke & Mosebach, 1975b). However, a total absence of labeled β -alanine was reported for rat blood plasma after injection of ^3H uridine (Dahnke & Mosebach, 1975b). In mouse, β -alanine + β -UIP accumulated not only in the liver but also in blood, indicating a certain transport of these substances out of the liver into the circulation.

The uneven distribution of volatile radioactivity between blood and liver until 60 min after injection was inconsistent with the very rapid distribution of $^3\text{H}_2\text{O}$ throughout the rat body (Dahnke & Mosebach, 1975b). Differences in injection and blood sampling techniques most probably explain this anomaly. Besides the divergent results reported for rat and mouse blood concerning labeled β -alanine and β -alanine + β -UIP, we found that the mouse liver, contrary to the rat liver, effectively incorporated radioactivity into uracil nucleotides and UDP-sugars (Tables 1 and 3) and that the mouse liver cells retained labeled uridine, uracil, and dihydrouracil during the complete pulse period. In the mouse liver, the level of ^3H uridine equaled or exceeded that of intraperitoneally sampled blood from about 10 min after injection. A certain contamination of the liver tissue by the intraperitoneally administered ^3H uridine and by uridine catabolic products might have occurred during the first 20 min after injection, because during this period unabsorbed radioactivity from ^3H uridine still remains in the peritoneal cavity (Yngner *et al.*, 1975; Engelbrecht *et al.*, 1977). However, from the radioactivity recovered in the nucleotides and UDP-sugars, it is evident that uridine was incorporated into the mouse liver cells. Very little labeled uridine, uracil, and uracil nucleotides were obtained in rat liver acid soluble fraction (Dahnke & Mosebach, 1975b; Yngner, unpublished results) probably because the ^3H -labeled metabolites were effectively catabolized to β -alanine and $^3\text{H}_2\text{O}$ on entry into the liver cells. Only 0.12% of the rat liver acid soluble fraction was recovered in UTP, whereas the corresponding value for mouse liver was 10% (Table 3). Thus the mouse liver cells seem to utilize the salvage pathway to a much greater extent than the rat liver cells. Several studies have shown a discrimination of uridine in favour of orotic acid for incorporation into rat liver (Witschi, 1972; Ord & Stocken, 1972; Dahnke, 1975a; Čihák *et al.*, 1972; Lewan, unpublished results), whereas uridine seems to be well utilized in mouse liver (Kochakian *et al.*, 1972; Etrychova & Jakoubek, 1975; Pakkenberg & Fog, 1972). A comparison of the percentage of administered ^3H uridine and ^{14}C orotic acid radioactivity incorporated into the acid soluble fraction of different mouse organs (Table 4) showed a close simi-

larity in utilization of the labeled substances, especially in the liver. Its comparatively low uridine orotic acid ratio 2 min after injection might indicate a slightly higher extraction rate of orotic acid than of uridine. A corresponding ratio calculated for radioactivity in the liver uracil nucleotides 10–60 min after ^3H uridine and ^{14}C orotic acid administration amounted to about 0.9 and indicated that the mouse liver utilized uridine and orotic acid to a similar extent. Moreover, analysis of radioactivity in liver acid soluble fraction, UTP, and RNA (Table 3) showed that the label from ^3H uridine was far more effectively incorporated into UTP and RNA of mouse liver than of rat liver. Ten % of the radioactivity in the mouse liver acid soluble fraction was recovered in UTP and 1.7% in RNA, whereas the corresponding values for rat liver was 0.12% and 0.03%, respectively. This species difference might be due to higher uridine kinase activity and lower phosphorylase activity in mouse liver compared with rat liver (Reichard & Sköld, 1958) as the size of the UTP pool ($\mu\text{mole/g wet wt}$) is almost identical in mouse and rat liver cells (Keppler *et al.*, 1970; Yngner, unpublished results). Uridine kinase activity is generally considered to reflect the relative efficiency of the cell or tissue in utilizing preformed pyrimidine precursors by the salvage pathway (Čihák *et al.*, 1974).

The comparatively low ratio of incorporated radioactivity from ^3H uridine compared with ^{14}C orotic acid in the acid soluble fraction of the mouse kidney is perhaps due to a large amount of the injected orotic acid being excreted unmetabolized into the urine (Hurlbert & Potter, 1954). It should be noted that the presented incorporation ratios are influenced by the overall metabolism in the animal and by tissue differences in metabolic activity and excretion capacity of especially $^3\text{H}_2\text{O}$ and $^{14}\text{CO}_2$. Thus the increased incorporation ratio calculated for mouse thymus and brain 60 min after injection might reflect such a condition, although rat brain was reported to utilize preformed pyrimidines to a much greater extent than it utilizes the *de novo* synthetic pathway to supply its requirements for pyrimidines (Hogans *et al.*, 1971; Pakkenberg & Fog, 1972).

Large differences between various RNA precursors concerning specificity for RNA labeling in different organs have been reported (Dahnke, 1975a). Compared to RNA from rat muscle and brain, the liver

Table 4. Ratio^a of incorporated radioactivity from ^3H uridine compared with ^{14}C orotic acid^b in the acid soluble fraction from mouse organs

Time after injection (min)	Liver	Kidney	Spleen	Thymus	Brain
2	0.53	0.14	1.03	1.81	0.50
10	1.15	0.13	0.87	0.38	0.86
60	0.95	0.08	0.59	2.86	3.00

^a The ratio was calculated as:

^3H -activity (counts/min per g wet wt) in the acid soluble fraction in % of totally injected ^3H -activity
 ^{14}C -activity (counts/min per g wet wt) in the acid soluble fraction in % of totally injected ^{14}C -activity

^b From Lewan *et al.* (1975).

RNA was heavily labeled with [^3H]orotic acid and much more specifically labeled with this precursor than with [^3H]uridine (Dahnke, 1975a; Lewan *et al.*, unpublished results). RNA radioactivity has often been analysed in the alkaline extracts of PCA extracted tissue homogenates obtained according to the Schmidt-Tannhauser method. This should be relevant only when specific RNA precursors are used (Hurlbert & Potter, 1952; Blobel & Potter, 1968; Bucher & Swaffield, 1969; Lewan *et al.*, 1975; Dahnke, 1975a) because large amounts of non-RNA associated radioactivity can be released during alkaline RNA hydrolysis. Our results indicate that, although uridine seems to be as well utilized as orotic acid for anabolism in the mouse liver, only about 15% of the ^3H -radioactivity in the alkaline extract emanated from RNA. Even more unspecific radioactivity was obtained from the rat liver. Similar results would probably be obtained from other tissues, but the percentage of unspecific label released during alkaline hydrolysis is possibly smaller in tissues that rely mainly on the salvage pathway for incorporation of labeled pyrimidine compounds into RNA.

The here presented data indicate differences in the importance of alternative synthetic pathways for nucleotide and RNA synthesis between mouse and rat. They also emphasize the importance of information about the specificity of labeled precursors for incorporation into nucleotides and nucleic acids and suggest that the calculation of the rate of RNA synthesis is based on the analysis of radioactivity incorporated into isolated RNA and on specific activity of the UTP-pool by use of more than one precursor.

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EFFECTS OF PARTIAL HEPATECTOMY ON RNA POLYMERASE ACTIVITIES
IN MOUSE LIVER

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ABSTRACT

1. Chromatin-bound and poly[d(A-T)]dependent RNA polymerase I plus III and II activities of mouse liver were analysed 24 h and 48 h after partial hepatectomy.
2. Chromatin-bound RNA polymerase I plus III activity showed an increase of 57% at 24 h and 51% at 48 h after partial hepatectomy.
3. There was a decrease in chromatin-bound RNA polymerase II activity of 15% at 24 h and 34% at 48 h after partial hepatectomy.
4. There were no significant changes in poly[d(A-T)]dependent RNA polymerase activities.
5. Heparin caused an approximately 10-fold increase in chromatin-bound RNA polymerase II activity. The stimulation by heparin was significantly increased 48 h after partial hepatectomy.
6. Anaesthesia and/or surgery had great influence on RNA polymerase activities. At 24 h after operation, chromatin-bound RNA polymerase I plus III and II activities were depressed, and the liver cell chromatin was more susceptible to stimulation by heparin.

INTRODUCTION

Regenerating liver is an often used model for studying metabolic processes during rapid proliferation (Bresnick, 1971; Bucher & Malt, 1971; Lesch & Reutter, 1975; Lewan et al., 1977). In rat liver after partial hepatectomy there is a rapid increase in RNA synthesis which can be demonstrated by incorporation of orotic acid into RNA (Bresnick, 1971; Bucher & Malt, 1971). Enhanced incorporation of orotate into RNA of regenerating liver is observed 3 h - 6 h after operation (Bucher & Swaffield, 1969), and the maximal rate of RNA synthesis occurs 12 h - 30 h after partial hepatectomy (Bresnick, 1971).

An examination of RNA polymerase activities 18 h after partial hepatectomy in the rat reveals increased levels of RNA polymerase activities in all three classes of RNA polymerase, i.e. RNA polymerase I, which is responsible for the synthesis of pre-ribosomal RNA, RNA polymerase II, which transcribes Hn RNA, and RNA polymerase III, which is involved in the synthesis of tRNA and 5 S RNA (Organtini et al., 1975; Schmid & Sekeris, 1975; Yu, 1975 b; Duceman & Jacob, 1980).

In mouse liver during regeneration, in contrast to the rat, no increase in RNA synthetic rate can be demonstrated by incorporation of either orotic acid (Yngner et al., 1979 a; Yngner et al., 1980) or uridine (Yngner et al., 1979 b) into RNA.

The aim of this study was therefore to determine whether the activities of transcriptionally active DNA dependent RNA polymerases were altered in mouse liver after partial hepa-

tectomy. If any alterations were found, it would be of interest to examine if the pattern of activity revealed by mouse liver differed from the pattern of activity demonstrated in regenerating rat liver.

Furthermore, we were interested in determining if the activities of nuclear free RNA polymerases were altered in the same manner as transcriptionally active RNA polymerases. It has been shown in regenerating rat liver that the two populations of RNA polymerase exist in a sensitive equilibrium (Yu, 1975 b). In other proliferative systems, however, the two populations react independently of each other (Hentschel & Tata, 1977; Tillyer & Butterworth, 1978).

The stimulatory effect of polyanions on nuclear transcription is well documented, and in such investigations the sulphated polysaccharide heparin has been frequently used (de Pomerai et al., 1974; Warnick & Lazarus, 1975; Coupar & Chesterton, 1977). Its effects have been described as an increase in the number of initiation sites (Warnick & Lazarus, 1975) or as increased elongation rate for RNA polymerase II (Coupar & Chesterton, 1977). Concomitant with the stimulatory effects on template properties, treatment with heparin results in considerable modification of the structure of chromatin (de Pomerai et al., 1974).

After partial hepatectomy a number of changes in chromatin properties take place. For example, liver cells stimulated to proliferate by partial hepatectomy show an increase in nuclear acid polysaccharide over resting liver cell nuclei, which contain mainly neutral polysaccharides (Ohnishi et al., 1973). Thus, there are normal changes in chromatin properties during regeneration, resembling the in vitro effects of heparin.

There is the possibility that chromatin in liver cells exposed to partial hepatectomy may be more susceptible to the stimulation of heparin than is chromatin in liver cells from unoperated or sham-operated controls. It was thus of interest to evaluate the effect of heparin on the capacity of chromatin for RNA synthesis following partial hepatectomy.

MATERIALS AND METHODS

Materials

Adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP), uridine triphosphate (UTP), phospho-creatine, creatine phosphokinase (E C 2.7.3.2), dithiotreitol, Tris base, Tris HCl and heparin were obtained from Sigma Chemical Co., St. Louis, Mo., USA; α -amanitin from Serva Entwicklungslabor, Heidelberg, Germany. [^3H]UTP (45 Ci/mmole) was purchased from the Radiochemical Centre, Amersham, Bucks., England; actinomycin D from Merck, Sharp and Dohne BV, Haarlem, The Netherlands; poly [d(A-T)] from Boehringer Mannheim, Germany, and glassfibre filters from Arthur H Thomas Co., Philadelphia, Pa., USA. The chemicals used were of analytical grade whenever possible.

Animals

Male NMRI mice (SPF quality, Anticimex, Sweden) weighing 26-28 g were used for the experiments. The animals were kept under constant conditions of light and temperature, and they had free access to food and water.

Partial hepatectomy

Partial hepatectomy was performed according to the method of Higgins and Anderson (Higgins & Anderson, 1931). In sham-operated animals the peritoneal cavity was opened and the liver lobes exposed. Operations were carried out under ether anaesthesia and took place between 9:00 and 11:00 am. The animals were killed at 24 h and 48 h after partial hepatectomy or sham-operation respectively.

Preparation of nuclei

Liver nuclei were prepared as described by Yu (Yu, 1975 a) in 2.3 M sucrose - 1 mM CaCl_2 . Isolated nuclei were suspended in chromatin medium (1 mM Tris-HCl, pH 8 at 25°C, 1 mM dithio-treitol and 4% glycerol) to give an A_{260} of 20 optical density (OD) units/ml. Approximately 19 OD units were obtained per g of liver wet weight after partial hepatectomy and 27 OD/g in sham-operated and unoperated controls.

Assay for chromatin-bound RNA polymerase activity

Approximately 2 OD units of liver nuclei were incubated in a total volume of 140 μl . The mixture contained 25 mM Tris-HCl buffer (pH 7.8 at 25°C), 2 mM MgCl_2 , 2 mM MnCl_2 , 5 mM dithio-treitol, 70 mM $(\text{NH}_4)_2\text{SO}_4$, 4% glycerol, 0.3 mM each of ATP, GTP and CTP, 0.3 mM $[^3\text{H}]\text{UTP}$ (125 mCi/mmol), 3 mM phosphocreatine and 0.25 U creatine phosphokinase. For inhibition of RNA polymerase II, 1.4 μg of α -amanitin/ml were included, and where appropriate 1 mg/ml of heparin was added (170 units/mg).

After incubation at 35°C for 20 minutes, 100 μl from each incubation was transferred to glass-fibre filters prepared with trichloroacetic acid. The filters were transferred to individual 25 ml E-flasks. Ice-cold 10% (W/V) trichloroacetic acid containing 1 mM AMP (10 ml) was added. After 15 min the solution was changed to 10 ml of 5% TCA containing 0.05 M $\text{Na}_4\text{P}_2\text{O}_7$. After gentle vibration for 15 min this step was repeated, followed by one extraction in 10 ml of 5% TCA for 15 min and then 3 x 10 ml of ethanol for 5 min each. The glass-fibre filters were dried and counted for radioactivity with Insta-gel scintillation fluid (Packard) in a Packard liquid scintillation counter.

Assay for poly[d(A-T)] dependent polymerase activity

Approximately 2 OD units of the liver nuclei were incubated in 148 μl . The mixture was as described above but with 35 mM

$(\text{NH}_4)_2\text{SO}_4$. Actinomycin D (50 $\mu\text{g}/\text{ml}$) was present, and α -amanitin (1.4 $\mu\text{g}/\text{ml}$) was used to inhibit free RNA polymerase II activity. The concentration of poly[d(A-T)] of 40 $\mu\text{g}/\text{ml}$ was used in the incubations, since poly[d(A-T)] was checked not to be rate-limiting at that concentration. The values of poly[d(A-T)] dependent RNA polymerase activity were divided by a factor of 2 in order to correct for the incorporation of UMP into poly (U-A). In poly (U-A) twice as many radioactively labelled UMP are incorporated per DNA template as into the average DNA template.

Evaluation of results

RNA polymerase II activity was estimated from the difference between total and RNA polymerase I plus III activity. Results were expressed as pmol UMP incorporated per mg of nuclear DNA. It was assumed that 21 OD units corresponded to 1 mg chromatin DNA. For each of the liver treatments a plot was made for pmole UMP incorporated into RNA to μg DNA added, to make sure that there was a linear relationship between the incorporation of UMP into RNA and the concentration of DNA up to the concentration used in the experiment. Statistical analysis of the data was performed according to analysis of variance (Snedecor & Cochran, 1973). In Table 4, however, analysis of variance was not applicable. Instead Kruskal-Wallis' test was used to test if a difference existed between any of the various treatments, and Wilcoxon-Mann-Whitney's test was used to test each group against the other (Siegel, 1956).

RESULTS

Chromatin-bound RNA polymerase activity

Table 1 shows the chromatin-bound RNA polymerase I plus III and polymerase II activity per mg of nuclear DNA. In this study the chromatin-bound RNA polymerase I plus III activity made up 60% of the total chromatin-bound activity in normal liver, and RNA polymerase II accounted for 40%. RNA polymerase I plus III revealed an increase in activity both 24 h and 48 h after partial hepatectomy compared to the unoperated controls. The increase was 35% and 53% at 24 h and 48 h respectively. Compared to sham-operated animals the rise in I plus III activity was 57% and 51% at 24 h and 48 h respectively. The sham operation depressed RNA polymerase I plus III activity 14% 24 h after operation. This effect was no longer seen 48 h after operation.

The activity of RNA polymerase II showed a decrease both 24 h and 48 h after partial hepatectomy compared to the unoperated controls. The decrease was 40% and 36% at 24 h and 48 h respectively. Compared to sham-operated controls the decrease was 15% and 34% at 24 h and 48 h respectively. Sham operation caused a 29% decrease in RNA polymerase II activity 24 h after operation, and in accordance with the results for RNA polymerase I plus III the depression was no longer seen 48 h after operation. Thus, both RNA polymerase I plus III and polymerase II activities were depressed as a result of the operation procedure.

Poly[d(A-T)] dependent RNA polymerase activity

Table 2 shows the poly[d(A-T)] dependent, or free, RNA polymerase I plus III and polymerase II activity per mg of nuclear DNA. The poly[d(A-T)] dependent RNA polymerase activity amounted to approximately 60% of the total RNA polymerase activity. There was a decrease in the activity of RNA polymerase I plus III both

24 h and 48 h after partial hepatectomy compared to unoperated controls. The decrease amounted to 14% and 40% at 24 h and 48 h respectively. Compared to sham-operated controls there was instead a 10% increase in RNA polymerase I plus III activity 24 h after partial hepatectomy. 48 h after partial hepatectomy, however, the RNA polymerase I plus III activity of regenerating liver was again lower than the RNA polymerase I plus III activity of sham-operated controls. The decrease amounted to 20%. Sham operation caused a depression of the poly[d(A-T)] dependent RNA polymerase I plus III activity both 24 h and 48 h after operation and the depression was 22% and 24% at 24 h and 48 h respectively.

The activity of the poly[d(A-T)] dependent RNA polymerase II showed a decrease at both 24 h and 48 h after partial hepatectomy compared to unoperated controls. The decrease was 44% and 55% at 24 h and 48 h respectively. Compared to sham-operated control animals the decrease amounted to 26% at 48 h. Sham operation also caused a depression of the poly[d(A-T)] dependent RNA polymerase II activity. The decrease in activity amounted to 48% and 39% at 24 h and 48 h respectively after partial hepatectomy.

To sum up, the chromatin-bound RNA polymerase I plus III activity was significantly increased after partial hepatectomy, whereas there was a decrease in RNA polymerase II activity (Table 1). In sham-operated animals both RNA polymerase I plus III and polymerase II showed a slight decrease compared to unoperated controls at 24 h, but the effect was no longer seen 48 h after operation.

The changes in poly[d(A-T)] dependent RNA polymerase activity caused by liver regeneration or by the operation procedure were not significant, but all operated animals revealed lower values

of activity compared to the unoperated controls (Table 2).

The relative proportions of RNA polymerase I plus III and II

Table 3 shows the ratio of RNA polymerase I plus III activity to total RNA polymerase activity of chromatin-bound and poly[d(A-T)] dependent RNA polymerase following partial hepatectomy or sham operation. The relative proportion of chromatin-bound RNA polymerase I plus III activity significantly increased both 24 h and 48 h after partial hepatectomy. In the poly[d(A-T)] dependent RNA polymerase activities, however, no relative changes between the different polymerases could be detected.

RNA polymerase II activity in the presence of heparin

Table 4 shows the chromatin-bound RNA polymerase II activity in the presence of heparin. The addition of heparin caused a heavy increase in RNA polymerase II activity. Compared to the activity of RNA polymerase II in the absence of heparin (Table 1), the activities showed an 8-fold to 13-fold increase after addition of heparin. The stimulation caused by heparin was significantly greater in regenerating livers and in sham-operated controls 24 h after operation than in unoperated controls. Thus, 24 h after operation the effect of heparin was just as great in regenerating liver as in sham-operated controls. 48 h after partial hepatectomy, however, the marked effect of heparin was still seen in the hepatectomized animals, whereas the sham-operated controls no longer revealed a value of stimulation above that of the unoperated controls.

The activities of RNA polymerase II in the presence of heparin did not differ significantly between any of the liver treatments. The stimulatory effect of heparin on RNA polymerase II activity, however, was significantly different after the various treatments.

The addition of heparin also caused an increase in RNA polymerase I plus III activity. The activity in the presence of heparin (not shown) amounted to approximately twice the value of RNA polymerase I plus III activity in the absence of heparin shown in Table 1. There were no significant differences between the liver treatments in stimulation by heparin of RNA polymerase I plus III activity.

DISCUSSION

During mouse liver regeneration no increased RNA synthesis can be demonstrated by incorporation of either orotic acid (Yngner et al., 1979 a; Yngner et al., 1980) or uridine (Yngner et al., 1979 b) into RNA. In rat liver during regeneration, however, there is a well documented increase in RNA synthesis (Bresnick, 1971; Bucher & Malt, 1971; Lewan et al., 1977). The enhanced rate of RNA synthesis has been demonstrated by incorporation of radioactively labelled precursors into RNA (Bucher & Swaffield, 1969) as well as by examination of RNA polymerase activities (Organtini et al., 1975; Schmid & Sekeris, 1975; Yu, 1975 b; Duceman & Jacob, 1980).

The discrepancy between regenerating mouse and rat liver may have several reasons. One possibility is that in mouse liver during regeneration there is actually no increased synthesis of RNA but the accretion of RNA found after partial hepatectomy (Lewan, 1972) is mainly due to a decreased breakdown of RNA. Another possibility is that the uptake of the precursor is influenced by the regenerative process, or liver regeneration leads to changes in size of one or several of the pyrimidine pools, through which the labelled precursor must pass. To elucidate the question of an increased RNA synthesis, this study on RNA polymerase activities was performed.

Chromatin-bound RNA polymerase activities

The results in this study showed that there was a distinct increase in chromatin-bound RNA polymerase I plus III activity during mouse liver regeneration. Both 24 h and 48 h after partial hepatectomy the activity was significantly above the corresponding activity of sham-operated controls. As a result of the enhanced activity there was an increase in the relative proportion of RNA polymerase

I plus III to 78-79% of total RNA polymerase activity during liver regeneration. This increase is practically identical with the values documented for rat liver (Organtini et al., 1975; Yu, 1975 b). The relative amount of RNA polymerase I plus III of 62% in control animals is also in agreement with what is found in rat liver (Yu, 1975 b) as well as in mouse liver (Näslund & von der Decken, 1981). The registered increase in RNA polymerase I plus III activity was probably an effect of changes in RNA polymerase I activity, since RNA polymerase III makes up only approximately 17% of the I plus III activity (Tata & Baker, 1978).

The rise in RNA polymerase I activity during mouse liver regeneration, found in this study, is in accordance with several observations on different tissues after varying physiological stimuli. In regenerating rat liver RNA polymerase I activity is enhanced 18 h after partial hepatectomy (Organtini et al., 1975; Schmid & Sekeris, 1975; Yu, 1975 b; Duceman & Jacob, 1980). Hydrocortisone stimulates RNA polymerase I activity in rat liver (Sajdel & Jacob, 1971), and the same effect is found for the stimulation of testosterone on mouse kidney (Jänne et al., 1976).

After stimulation of liver cells by hydrocortisone the increased RNA polymerase activity is exclusively in polymerase I (Sajdel & Jacob, 1971). In regenerating rat liver, however, an increase is also documented for RNA polymerase II and III, though the most pronounced increase is in polymerase I (Duceman & Jacob, 1980). Since there is an elevated RNA polymerase II activity during rat liver regeneration 18 h after partial hepatectomy (Duceman & Jacob, 1980), we had expected to find a rise in this enzyme activity in mouse liver 24 h after partial hepatectomy. However, no such increase could be demonstrated. Instead, 24 h as well 48 h after partial hepatectomy there was a decrease in

RNA polymerase II activity compared to sham-operated controls. The possibility cannot be excluded, however, that an increased transcription of RNA polymerase II had already taken place during the regenerative period up to 24 h and was not detected by studying the conditions 24 h and 48 h after partial hepatectomy.

In a series of Morris rat hepatomas, RNA polymerase I activity has been shown to be elevated relative to the value of this enzyme activity in normal, resting liver. Furthermore, the RNA polymerase I activity correlates well with the growth rate of the tumours (Duceman & Jacob, 1980). No significant changes in RNA polymerase II activity were found in any of the tumours examined.

Thus, during rapid proliferation there is a demand for high RNA polymerase I activity but not necessarily for an enhanced RNA polymerase II activity. Still, there must take place qualitative changes in the transcription activity of RNA polymerase II during development from normal liver to hepatoma. The same discussion may be applied to the induction of mouse liver regeneration. This process leads to an increased production of histones, which is correlated with DNA synthesis (Kuehl, 1979). In mouse liver the onset of DNA synthesis is in the interval 24 h to 48 h after partial hepatectomy (Lewan et al., 1977). Thus, during the regenerative period when RNA polymerase II activity was found to be depressed, the transcription of some mRNA species, with low levels in normal, resting liver, are likely to be increased. These facts indicate that a considerable proportion of liver proteins synthesized under normal conditions must be depressed.

Poly[d(A-T)] dependent RNA polymerase activities

The increase in chromatin-bound RNA polymerase I activity during regeneration was not accompanied by a similar rise in poly[d(A-T)] dependent, or free, RNA polymerase I activity. Instead, after partial hepatectomy, the activities of both poly[d(A-T)] dependent RNA polymerase I plus III and II were at the same level or beneath the level of sham-operated animals.

In contrast to our results on mouse liver, the preferential increase in RNA polymerase I activity expressed by chromatin-bound RNA polymerase in regenerating rat liver is accompanied by a selective stimulation also of free, or poly[d(A-T)] dependent, RNA polymerase I. Both free and chromatin-bound activities revealed an increase of 100-200% (Yu, 1975 b). Thus, in regenerating rat liver there exists an equilibrium between the free enzyme and the engaged enzyme in the nuclei, which cannot be demonstrated in mouse liver.

In other proliferating systems, besides regenerating mouse liver, the two populations of RNA polymerases have been shown to respond independently of each other. Systems, where the free RNA polymerase activity responds independently of the chromatin-bound activity, are for example lymphocytes stimulated by PHA (Tillyer & Butterworth, 1978) and the stimulation of dormant Artemia (Hentschel & Tata, 1977).

Our results underline the differences between mouse and rat liver regeneration previously pointed out by Yngner (Yngner et al., 1979 a; Yngner et al., 1979 b; Yngner et al., 1980). Total RNA polymerase activity showed no increase, and RNA polymerase II rather showed a reduced activity, in contrast to what has been demonstrated during rat liver regeneration. Nuclear free RNA polymerases were not altered in the same manner as transcriptionally

active RNA polymerases, as was the case in the rat. These discrepancies between mouse and rat liver cannot be ascribed solely to the prolongation of the regenerative process found in mouse liver (Heby & Lewan, 1971; Lewan, 1972; Lewan et al., 1977) but rather reflect different ways of providing for the greater RNA content needed during rapid proliferation.

Influence of the operation procedure

Anaesthesia and/or surgery had a great influence on RNA polymerase activity, as revealed by the sham-operated controls. At 24 h after operation a depression was seen in chromatin-bound RNA polymerase I plus III and II activity. At 48 h after operation the activities were completely restored to levels revealed by the unoperated controls. The poly[d(A-T)] dependent RNA polymerase activities of sham-operated controls were influenced in the same direction as the chromatin-bound activities by the operation procedure. However, these RNA polymerase activities were not recovered by 48 h.

Effect of heparin

The stimulatory effect of heparin on transcription activity was considerably greater in regenerating liver and in liver from sham-operated animals at 24 h than in liver from sham-operated animals at 48 h or from unoperated controls. At 24 h after operation, the influence of anaesthesia and/or surgery resulted in a liver cell chromatin more susceptible to stimulation by heparin. By 48 h the chromatin in liver nuclei of sham-operated animals was no longer stimulated above the level of unoperated controls, while the most pronounced increase in RNA polymerase II activity was expressed 48 h after partial hepatectomy. Thus, the changes in chromatin properties were results of the operation procedure as well as of the cellular re-programming to proliferation.

After addition of heparin, RNA polymerase II activity was enhanced to an optimal level, which was approximately the same for the different groups of animals regardless of pretreatment. The greater stimulation of template activity caused by heparin after partial hepatectomy and 24 h after sham operation was a consequence of the depressed activity of RNA polymerase II, by these treatments in the absence of heparin.

The stimulatory action of heparin on chromatin template activity can be ascribed to an interaction with the chromatin and not with the polymerase (Warnick & Lazarus, 1975). The interaction has been suggested to lead to optimal conditions for the propagation of RNA molecules, i.e. an increased elongation rate (Coupar & Chesterton, 1977). Another possibility is that inactive or blocked transcription complexes are activated by heparin, resulting in an increased number of initiation sites activated for RNA polymerase II, and an increased number of RNA chains synthesized (Warnick & Lazarus, 1975; de Pomerai et al., 1974; von der Decken & Åström, 1981). The analysis made in this study did not discriminate between these theories of heparin action.

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Table 1.

Chromatin-bound RNA polymerase activity per mg of nuclear DNA.

Animal treatment	Time after operation (h)	RNA polymerase			
		I + III		II	
		pmol UMP per mg nuclear DNA	% of unoperated control	pmol UMP per mg nuclear DNA	% of unoperated control
Unoperated control	-	175 $\pm$ 12 ^{a,c}	100	112 $\pm$ 16 ^{e,f,i}	100
Sham-operated control	24	150 $\pm$ 14 ^b	86	79 $\pm$ 8 ^{h,i}	71
Partial hepatectomy	24	236 $\pm$ 19 ^{a,b}	135	67 $\pm$ 7 ^e	60
Sham-operated control	48	177 $\pm$ 15 ^d	101	110 $\pm$ 12 ^{g,h}	98
Partial hepatectomy	48	268 $\pm$ 16 ^{c,d}	153	72 $\pm$ 9 ^{f,g}	64

The results are the mean values $\pm$ SEM of 12 preparations with at least 2 determinations for each preparation. Results with the same superscript are significantly different.

e,f,g,h,i p < 0.05

a p < 0.01

b,c,d p < 0.001

Table 2.

Poly[d(A-T)] dependent RNA polymerase activity per mg of nuclear DNA.

Animal treatment	Time after operation (h)	RNA polymerase			
		I + III		II	
		pmol UMP per mg nuclear DNA	% of unoperated control	pmol UMP per mg nuclear DNA	% of unoperated control
Unoperated control	-	250 [±] 41	100	186 [±] 42	100
Sham-operated control	24	194 [±] 36	78	96 [±] 25	51
Partial hepatectomy	24	214 [±] 22	86	103 [±] 14	56
Sham-operated control	48	189 [±] 20	76	113 [±] 22	61
Partial hepatectomy	48	151 [±] 24	61	84 [±] 30	45

The results are the mean values $\pm$ SEM of 5-6 preparations with at least 2 determinations for each preparation. The figures have been divided by a factor of 2 in order to correct for the incorporation of UMP into poly(U-A).

Table 3.

Relative proportions of the nuclear RNA polymerase activities following partial hepatectomy.

Animal treatment	Time after operation (h)	RNA polymerase I + III activity/ total activity	
		Chromatin-bound activity	Poly[d(A-T)] dependent activity
Unoperated control	-	0.62 ^{a,b}	0.59
Sham-operated control	24	0.65 ^c	0.69
Partial hepatectomy	24	0.78 ^{a,c}	0.68
Sham-operated control	48	0.62 ^d	0.64
Partial hepatectomy	48	0.79 ^{b,d}	0.69

The results are the mean values $\pm$ SEM of 12 preparations for chromatin-bound activity and 5-6 preparations for poly[d(A-T)] dependent activity. At least 2 determinations were made for each preparation. Results with the same superscript are significantly different.

a,b,c,d $p < 0.001$

Table 4

Chromatin-bound RNA polymerase II activity after stimulation by heparin.

Animal treatment	Time after operation (h)	Activity after addition of heparin	Activity, heparin present/ activity, heparin absent
		pmol UMP per mg nuclear DNA	
Unoperated control	-	874 [±] 107	8.2 ^{b,c,d}
Sham-operated control	24	928 [±] 105	12.6 ^a
Partial hepatectomy	24	833 [±] 78	12.7 ^b
Sham-operated control	48	697 [±] 84	6.4 ^{a,c,e}
Partial hepatectomy	48	974 [±] 127	13.9 ^{d,e}

Chromatin-bound RNA polymerase II activity was measured after addition of heparin (first column). These figures were then divided by the RNA polymerase II activities measured in the absence of heparin, given in Table 1 (second column). The results are the mean values $\pm$ SEM of 12 preparations with at least 2 determinations for each preparation. Results with the same superscript are significantly different.

c p < 0.05

a p < 0.01

b,d,e p < 0.001

RIBONUCLEIC ACID SYNTHESIS OF REGENERATING MOUSE LIVER EVALUATED
BY INCORPORATION OF [METHYL- ^{14}C]METHIONINE AND [^{14}C] OROTIC ACID
AND BY DETERMINATION OF RNA POLYMERASE ACTIVITY.

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SYNOPSIS

In the present investigation the synthesis of RNA during mouse liver regeneration was studied by different methods at 24 h and 48 h after partial hepatectomy. Total chromatin-bound RNA polymerase activity showed an increase of 32% at 24 h after partial hepatectomy. At 48 h a slight increase in total activity was also observed in regenerating liver, but the difference was not significant. The increase in total RNA polymerase activity was due to a rise in RNA polymerase I plus III activity. This enzyme activity was increased both 24 h and 48 h after partial hepatectomy. The increase was 57% at 24 h and 51% at 48 h and most certainly reflected an augmented transcription of rRNA during liver regeneration. When [methyl- ^{14}C]methionine was used for labelling of methyl groups in rRNA, there was an increased specific radioactivity of this class of RNA at both 24 h and 48 h. The increase was 263% and 103% at 24 h and 48 h respectively. Incorporation of [^{14}C]orotic acid resulted in an increased specific radioactivity of total RNA of 59% at 24 h, when a labelling time of 120 min was used. At 48 h after partial hepatectomy, however, no increased specific radioactivity of RNA could be demonstrated. The results in this study were discussed in relation to previous results from this laboratory on incorporation of orotic acid during mouse liver regeneration, where no increased incorporation of orotic acid could be demonstrated at 6 h or 24 h after partial hepatectomy. The results were also compared to the well documented increase in RNA transcription during rat liver regeneration.

INTRODUCTION

Numerous observations have been published dealing with different biochemical aspects of liver regeneration after partial hepatectomy. The regenerating rat liver is often used as a model for the study of molecular events during proliferation (Bresnick, 1971; Bucher & Malt, 1971; Lesch & Reutter, 1975; Lewan et al., 1977) and as a control tissue in studies of hepatoma growth (Weber et al., 1965; Lea et al., 1974; Duceman & Jacob, 1980).

There is an enhanced uptake and incorporation of labelled orotic acid into rat liver RNA early after partial hepatectomy (Bresnick, 1971; Bucher & Malt, 1971). A nearly 100% increase of the incorporation of orotic acid into the total acid soluble fraction and into UTP is observed at 24 h after operation (Yngner et al., 1979a). Since the specific radioactivity of RNA is increased more than that of UTP, there are strong indications of an enhanced RNA synthesis (Yngner et al., 1979a). This interpretation is supported by the fact that the activities of RNA polymerase I, II and III are elevated at 18 h after partial hepatectomy (Organtini et al., 1975; Yu, 1975b; Duceman & Jacob, 1980). Polymerase I is responsible for the synthesis of pre rRNA, polymerase II produces hnRNA, and polymerase III catalyzes the synthesis of pre tRNA and 5S RNA.

In another proliferating system, the rat hepatoma, the uptake and incorporation of orotic acid into RNA is decreased when compared to both regenerating liver and host liver (Weber et al., 1965; Lea et al., 1974). Furthermore, during hepatoma growth, only RNA polymerase I activity is enhanced in comparison to normal, resting liver (Duceman & Jacob, 1980).

In contrast to regenerating rat liver, mouse liver reveals no enhanced uptake of orotic acid or incorporation of the precursor into either UTP or RNA at 6 h and 24 h after partial hepatectomy (Yngner et al., 1979a; Yngner et al., 1980). Nor is there an increased incorporation of labelled uridine into UTP or RNA of regenerating mouse liver, despite an increased uptake of the precursor into the total acid soluble fraction (Yngner et al., 1979b).

An experimental system with many similarities to the regenerating liver is the kidney after unilateral nephrectomy. In this system, the labelling of RNA also seems to differ between mouse and rat. As in regenerating rat liver, there is an increased incorporation of orotic acid also in rat kidney RNA during compensatory hypertrophy (Cortes et al., 1976). In mouse kidney after unilateral nephrectomy, however, no increase in incorporation of uridine or orotic acid into RNA has been demonstrated (Hill et al., 1974). If methionine is used as labelled precursor instead, there is a substantial increase in the labelling of ribosomal RNA (rRNA) of the hypertrophying mouse kidney. This increase has been ascribed to a greater conservation of ribosomal precursor RNA (Hill et al., 1974).

The fact that no increased incorporation into RNA of either orotic acid or uridine has been demonstrated in mouse liver after partial hepatectomy may be interpreted as indicating that there is no increased RNA synthesis. However, there is obviously a rise in the RNA content during the regenerative process in mouse liver (Lewan, 1972). Thus, either transcription of RNA must be increased, though it is not detected by use of labelled orotic acid or uridine, or an increased conservation of RNA must occur. The present investigation was undertaken to elucidate the question of a possible increase in transcription during mouse

liver regeneration. Consequently, RNA synthesis was determined by different methods: (a) The synthesis of ribosomal RNA was studied by use of [methyl- ^{14}C] labelled methionine. One advantage of using methionine instead of a pyrimidine precursor is that its incorporation into RNA is not influenced by possible changes in size of the pyrimidine pools. (b) The rate of RNA transcription was also evaluated by determination of RNA polymerase activity. It was of interest to know whether regenerating mouse liver resembled rat liver after partial hepatectomy as regards the pattern of RNA polymerase activity, or whether the pattern of activity revealed by the hepatoma might be expressed also in a non-tumour system (Duceman & Jacob, 1980).

Both methods described revealed an increased synthesis of rRNA during mouse liver regeneration. (c) It therefore seemed justified to further evaluate the effect of partial hepatectomy on incorporation of orotic acid into mouse liver RNA to complete previous results from this laboratory (Yngner *et al.*, 1979a; Yngner *et al.*, 1980). In order to obtain incorporation into rRNA we utilized a relatively long labelling time, 120 min.

MATERIALS AND METHODS

Materials

[6- ^{14}C]Orotic acid (61 mCi/mmol), L-[methyl- ^{14}C]methionine (60.2 mCi/mmol) and [5,6- ^3H]uridine 5'-triphosphate (45 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, Bucks., England. Adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP), uridine triphosphate (UTP), phosphocreatine, creatine phosphokinase (EC 2.7.3.2), dithiotreitol, Tris

base and Tris HCl were obtained from Sigma Chemical Co., St. Louis, Mo., USA; α -amanitin from Serva Entwicklungslabor, Heidelberg, Germany, and glassfibre filters from Arthur H Thomas Co., Philadelphia, Pa., USA. The chemicals used were of analytical grade whenever possible.

Animals

Male NMRI mice (SPF quality, Anticimex, Sweden) weighing 26-28 g were used for the experiments. The animals were kept under constant conditions of light and temperature, and they had free access to food and water.

Partial hepatectomy

Partial hepatectomy was performed according to the method of Higgins and Anderson (Higgins & Anderson, 1931). In sham-operated animals the peritoneal cavity was opened and the liver lobes exposed. Operations were carried out under ether anaesthesia and took place between 9:00 and 11:00 am. The animals were killed at 24 h and 48 h after partial hepatectomy or sham-operation respectively.

IN VIVO EXPERIMENTS

Radioactive labelling

The animals received an i.p. injection of 6 μ Ci of L-[methyl- 14 C]-methionine dissolved in 90 μ l 0.9% NaCl or of 1.5 μ Ci of [6- 14 C]orotic acid dissolved in 75 μ l 0.9% NaCl. Determinations of radioactivity were carried out with Insta-gel scintillation fluid (Packard) in a Packard liquid scintillation counter.

Tissue sampling

Animals were killed 120 min after injection of [^{14}C]orotic acid and 180 min after injection of [methyl- ^{14}C]methionine. At 90 s prior to liver excision, the animals were anaesthetized in an ether/oxygen atmosphere (Bucher & Swaffield, 1966). Blood was collected from the brachial vessels, whereafter the liver was immediately excised and clamp-frozen in a metal tong precooled in liquid nitrogen. Both blood and liver were rapidly immersed into liquid nitrogen. Tissues were kept frozen until analysis.

Analysis of acid soluble fraction and of total RNA

Frozen liver was pulverized under liquid nitrogen and homogenized in 10 ml of ice-cold 0.4 M HClO_4 per g wet weight. After centrifugation the supernatant was adjusted to pH 8.0 with 7 M KOH/2 M KHCO_3 . The precipitated KClO_4 was sedimented by centrifugation. Aliquots of the supernatant were used for determination of the radioactivity of the total acid-soluble fraction. The radioactivity of UTP of the acid-soluble fraction was determined after thin-layer chromatography on pre-coated poly(ethyleneimine)-impregnated thin-layer plates. UTP was isolated by one-dimensional chromatography after elution in 1.6 M ammonium formate buffer, pH 3.5, to 3 cm from the point of application, followed by elution in 3.2 M ammonium formate buffer, pH 3.5, to the upper edge of the chromatogram. The UTP spot was identified under UV light after co-chromatography with the corresponding unlabelled marker compound. UTP was eluted from the cellulose with 0.7 M MgCl_2 /20 mM Tris-HCl, pH 7.4. The eluate was used for determination of the radioactivity of UTP.

The acid-insoluble material obtained after homogenization in 0.4 M HClO_4 and subsequent centrifugation was analyzed for RNA

according to the Schmidt-Tannhauser method as modified by Munro & Fleck (1966). Radioactivity was measured in aliquots from the RNA-fraction. DNA was assayed by the method of Burton (1956).

Extraction of rRNA

Ribosomal RNA was extracted according to the method of Parish and Kirby as modified by Axelsson et al. (1978). Liver tissue, 1 g, was homogenized with 20 ml of a solution containing 6% sodium 4-amino-salicylate (PAS), 6% butanol (v/v), 1% NaCl and 1% tri-isopropyl-naphtalene sulphonate (TIPNS). Then 13 ml of a phenol solution (500 g phenol, 70 ml m-cresol, 0.5 g 8-hydroxyquinoline and 55 ml water) was added. The mixture was stirred for 30 min at room temperature before centrifugation at 10 000 g for 10 min. The upper aqueous phase was removed, and to the phenol phase 20 ml of the solution (PAS-butanol-NaCl-TIPNS) was added. After stirring for 15 min the phases were separated by centrifugation at 10 000 g for 10 min. The aqueous phases were combined, and the solution was made 3% in NaCl. Half a volume of the phenol solution was added and the mixture was stirred for 15 min. After centrifugation for 10 min at 10 000 g the aqueous phase was collected and two volumes of ethanol containing 10% m-cresol (v/v) were added. RNA and DNA were allowed to precipitate at -20°C for 1 h. The precipitate was washed at $+4^{\circ}\text{C}$ with 2% sodium acetate in 75% ethanol and was then dissolved in 0.1 M sodium acetate, pH 6.0. The solution was made 4 M in NaCl, and RNA was allowed to precipitate over night at $+4^{\circ}\text{C}$. After centrifugation the RNA pellet was washed with 4 M NaCl in 0.1 M sodium acetate and dissolved in 0.5 ml Tris-buffer, 0.1 M, pH 7.0. For determination of RNA content and radioactivity the isolated rRNA was further analysed according to the method of Munro & Fleck (1966).

IN VITRO EXPERIMENTS

Preparation of nuclei

Liver nuclei were prepared as described by Yu (Yu, 1975a) in 2.3 M sucrose - 1 mM CaCl_2 . Isolated nuclei were suspended in chromatin medium (1 mM Tris-HCl, pH 8 at 25°C, 1 mM dithiotreitol and 4% glycerol) to give an A_{260} of 20 optical density (OD) units/ml. Approximately 19 OD units were obtained per g of liver wet weight after partial hepatectomy and 27 OD/g in sham-operated and unoperated controls.

Assay for chromatin-bound RNA polymerase activity

Approximately 2 OD units of liver nuclei were incubated in a total volume of 140 μl . The mixture contained 25 mM Tris-HCl buffer (pH 7.8. at 25°C), 2 mM MgCl_2 , 2 mM MnCl_2 , 5 mM dithiotreitol, 70 mM $(\text{NH}_4)_2\text{SO}_4$, 4% glycerol, 0.3 mM each of ATP, GTP and CTP, 0.3 mM $[^3\text{H}]\text{UTP}$ (125 mCi/mmol), 3 mM phosphocreatine and 0.25 U creatine phosphokinase. For inhibition of RNA polymerase II, 1.4 μg of α -amanitin/ml was included.

After incubation at 35°C for 20 min, 100 μl from each incubation was transferred to glass-fibre filters prepared with trichloroacetic acid. The filters were transferred to individual 25 ml E-flasks. Ice-cold 10% (w/v) trichloroacetic acid containing 1 mM AMP (10 ml) was added. After 15 min the solution was changed to 10 ml of 5% TCA containing 0.05 M $\text{Na}_4\text{P}_2\text{O}_7$. After gentle vibration for 15 min this step was repeated, followed by one extraction in 10 ml of 5% TCA for 15 min and then 3 x 10 ml of ethanol for 5 min each. The glass-fibre filters were dried and counted for radioactivity with Insta-gel scintillation fluid (Packard) in a Packard liquid scintillation counter.

Evaluation of results

Results of RNA polymerase activity were expressed as pmol UMP

incorporated per mg of nuclear DNA. It was assumed that 21 OD units corresponded to 1 mg chromatin DNA. For each of the liver treatments care was taken to ensure that there was a linear relationship between the incorporation of UMP into RNA and the concentration of DNA up to the concentration used in the experiment.

Statistical analysis of the data was performed according to Student's t-test.

RESULTS

Incorporation of [methyl- ^{14}C]methionine into ribosomal RNA

Table 1 shows the specific radioactivity of rRNA after labelling with [methyl- ^{14}C]methionine for 180 min. The incorporation of radioactively labelled methyl groups was increased at 24 h and 48 h after partial hepatectomy. The increase in specific radioactivity of rRNA of regenerating liver was 263% and 103% of the values of sham-operated controls at 24 h and 48 h respectively. Compared to unoperated controls the increase was even more pronounced.

RNA polymerase activity

Table 2 shows the total chromatin-bound RNA polymerase activity and polymerase I plus III activity per mg of nuclear DNA. There was an increase of 32% in total RNA polymerase activity 24 h after partial hepatectomy compared to the value of sham-operated controls. At 48 h an increase in total RNA polymerase activity of 18% was found, but the difference was not significant. Compared to unoperated controls no significant rise in total activity was observed at 24 h or 48 h.

RNA polymerase I plus III was increased both 24 h and 48 h after partial hepatectomy. The increase was 57% and 51% of the value of sham-operated controls at 24 h and 48 h respectively. Compared to unoperated controls the rise in I plus III activity was 35% ($p < 0.05$) and 53% ($p < 0.001$) at 24 h and 48 h respectively.

The sham-operation depressed total RNA polymerase activity 20% and RNA polymerase I plus III activity 14% at 24 h after operation. This effect was no longer seen 48 h after operation.

Incorporation of [^{14}C]orotic acid

Table 3 shows the radioactivity of total RNA, UTP and total acid soluble fraction of mouse liver after labelling with [^{14}C]orotic acid for 120 min. The specific radioactivity of liver RNA 24 h after partial hepatectomy was significantly increased (59%) compared to what was registered after sham operation. After 48 h, however, no increased specific radioactivity of RNA could be demonstrated in regenerating liver. The increase at 24 h was statistically significant also when the incorporation into RNA was related to g of liver or to mg of DNA (not shown). At 48 h there was no significant difference when the incorporation into RNA was related to these parameters. Compared to unoperated controls a slight increase was observed at 24 h and 48 h after partial hepatectomy, when the incorporation into RNA was related to these parameters.

The incorporation of orotic acid into the total acid soluble fraction of regenerating mouse liver did not differ significantly from the value obtained after sham operation. Compared to unoperated controls the incorporation was even slightly decreased. After 48 h the incorporation was decreased in regenerating liver both compared to sham-operated and unoperated controls.

The relative changes of incorporation into UTP after partial hepatectomy or sham-operation, compared to the values of unoperated controls, were quite in accordance with the differences between the total acid soluble fractions. Thus, the radioactivity of UTP was close to 10% of the total acid soluble radioactivity, irrespective of previous treatment.

Sham-operation depressed RNA specific radioactivity 35% ($p < 0.001$), radioactivity of UTP 22% and that of acid soluble fraction 25% ($p < 0.01$) at 24 h after partial hepatectomy.

When the incorporation into RNA was related to the incorporation into the acid soluble fraction (cpm of RNA:cpm of acid soluble fraction) there was a significantly increased incorporation at both 24 h (0.331 ± 0.007 in regenerating liver compared to 0.244 ± 0.019 in sham-operated control) and 48 h (0.378 ± 0.024 in regenerating liver compared to 0.225 ± 0.012 in sham-operated control). The value of unoperated control was 0.243 ± 0.010 . This relative incorporation of orotic acid into RNA of regenerating liver was 1.4 and 1.7 times the corresponding values of sham-operated controls at 24 h and 48 h respectively.

DISCUSSION

In the rat, as well as in the mouse, there is an increase of the RNA content of the liver during the regenerative process (Bresnick, 1971; Lewan, 1972). Consequently an increased transcription must take place and/or there must be a decreased degradation of RNA. In rat liver an increased transcription has been demonstrated by incorporation of pyrimidine precursors (Bresnick, 1971; Bucher & Malt, 1971; Lewan et al., 1977) as well as by studies of RNA polymerase activities during regeneration (Organtini et al., 1975; Schmid & Sekeris, 1975; Yu, 1975b; Duceman & Jacob, 1980).

When, in the present study, the synthesis of rRNA in regenerating mouse liver was examined by use of [methyl- ^{14}C]methionine, an increased incorporation into this class of RNA was revealed at both 24 h and 48 h after partial hepatectomy. Furthermore, RNA polymerase activities supported the interpretation of increased transcription of rRNA, i.e. the activity of RNA polymerase I plus III was elevated at both 24 h and 48 h after partial hepatectomy. Since RNA polymerase III makes up only approximately 17% of the I plus III activity (Tata & Baker, 1978), the results most certainly reflect an increase in the synthesis of rRNA.

In previous investigations from this laboratory it has been shown that the incorporation of [^{14}C]orotic acid into RNA, UTP and into the total acid soluble fraction is not increased over the values of normal and sham-operated controls at 6 h and 24 h after partial hepatectomy (Yngner et al., 1979a; Yngner et al., 1980). Nor is there an increased incorporation into mouse liver UTP and RNA at these times after partial hepatectomy when labelled uridine is used as precursor (Yngner et al., 1979b). Thus, even though there is an increased transcription of rRNA during mouse liver regeneration, as shown in this study, no increased specific radioactivity of total RNA has been demonstrated by labelling with orotic acid for either 20, 30 or 300 min (Yngner et al., 1979a; Yngner et al., 1980).

In the present study an unaltered uptake of orotic acid into the acid soluble fraction was found at 24 h after partial hepatectomy, when we utilized a labelling time of 120 min. At 48 h the uptake was reduced compared to the values of both unoperated and sham-operated controls. An increased specific radioactivity of RNA relative to sham-operated controls was found 24 h after partial hepatectomy. Relative to unoperated controls, however, the specific radioactivity was not increased at 24 h. In regenerating liver at 48 h the specific radioactivity of RNA was decreased both compared to sham-operated and unoperated controls. The low labelling at 48 h seemed to reflect changes in the uptake of the precursor.

The radioactivity of the acid soluble fraction of the liver should represent the amount of labelled precursor being transported into and metabolized in the liver cells. If the incorporation into RNA was corrected for changes in the uptake of the precursor, i.e. for changes in the total acid soluble fraction or for that of UTP, we found an increased labelling of RNA at both 24 h and 48 h after partial hepatectomy in comparison with unoperated and sham-operated controls. The incorporation into RNA in relation to the acid soluble fraction of regenerating liver was 1.4 and 1.7 times the control values of sham-operated animals at 24 h and 48 h respectively. Thus, only when differences in the uptake of the precursor were taken into account was there an agreement between the experiments in which labelled orotic acid was utilized and those in which incorporation of methionine and RNA polymerase activities were studied. However, the labelling of RNA after 120 min should mainly reflect the conditions in the acid soluble fraction and UTP early after injection of the precursor and not the state at the time of liver sampling.

In rat liver the UTP pool is known to expand early after partial hepatectomy (Bucher & Swaffield, 1969; Glazer, 1973), and for calculation of RNA synthesis the incorporation into RNA should be corrected for the specific radioactivity of UTP. Previous results on mouse liver, however, showed that there were no changes in the total UTP pool that could account for the fact that incorporation of orotic acid into RNA was not increased (Yngner et al., 1979a). The evaluation of the results is complicated even further, however, by the fact that there seems to be a compartmentation of the nucleotide pools, i.e. only a small part of the total cellular UTP is the immediate source for RNA synthesis (Wiegers et al., 1976; Genchev et al., 1980; Keppler & Holstege, 1982).

Rat liver is extracting orotic acid far more effectively than mouse liver (Yngner et al., 1979a; Durschlag & Robinson, 1980). During rat liver regeneration the incorporation of orotic acid is even more accentuated (Ord & Stocken, 1972), a fact that may be due to an activated transport of the precursor into the cells. In mouse liver no accentuated transport of orotic acid into the liver after partial hepatectomy has been found (Yngner et al., 1979a). In rat hepatoma it has been suggested that an impaired transport of orotic acid across the membrane may explain the low rate of incorporation of the precursor into the acid soluble fraction and into RNA when compared to regenerating liver and to host liver (Weber et al., 1965; Matsudaira et al., 1968; Lea et al., 1974).

The different pictures revealed by mouse liver and rat liver when labelled orotic acid is used as precursor may depend on the fact that the de novo pathway is the dominating route for pyrimidine nucleotide synthesis in the rat (Yu & Feigelson, 1970; Hogans et al., 1971; Witschi, 1972), whereas in mouse liver both the de novo and the salvage pathways for synthesis of nucleotides and RNA are well utilized (Yngner et al., 1977).

Furthermore, compartmentation of the nucleotide pools may lead to different utilization of orotic acid in mouse liver compared to rat liver. It has been proposed that hn RNA and pre rRNA are synthesized from partly independent nuclear pyrimidine pools (Genchev et al., 1980). This suggestion is based on the findings that hn RNA tends rather to be labelled by orotic acid whereas the synthesis of pre rRNA tends rather to be supplied by UTP labelled by incorporation of uridine. Since there is a considerable increase in the synthesis of hn RNA during rat liver regeneration but not during mouse liver regeneration at 24 h and 48 h, the utilization of orotic acid for RNA synthesis should not be expected to be the same during regeneration in these two species.

Also when RNA polymerase activities are concerned, the regenerating rat liver differs from regenerating mouse liver and from hepatoma. In mouse liver during regeneration, RNA polymerase I was elevated whereas RNA polymerase II was not. In rat liver all three major classes of RNA polymerase activities have been shown to be increased during regeneration, though RNA polymerase I was dominating (Organtini et al., 1975; Schmid & Sekeris, 1975; Yu, 1975b; Duceman & Jacob, 1980). The rise in RNA polymerase I activity during regeneration is in accordance with several observations on different tissues after varying physiological stimuli. Hydrocortisone stimulates RNA polymerase I activity in rat liver (Sajdel & Jacob, 1971) and the same effect is found for the stimulation of testosterone on mouse kidney (Jänne et al., 1976). Furthermore, in a series of Morris rat hepatomas, RNA polymerase I has been shown to be elevated relative to the value of this enzyme in normal, resting liver. No significant changes in RNA polymerase II activity were found in any of the tumours examined (Duceman & Jacob, 1980). Thus, it can be concluded that the pattern of RNA polymerase activities found in some hepatomas, i.e. increased activity of RNA polymerase I but not of polymerase II, is not restricted to neoplastic growth. A proliferating tissue such as mouse liver during regeneration revealed the same pattern.

This investigation underlined the differences indicated earlier between rat liver and mouse liver regarding utilization of orotic acid during regeneration (Yngner et al., 1979a; Yngner et al., 1980). It further revealed clear differences between the species in RNA polymerase activities during regeneration. There are thus differences between the species in their ways of providing for the increasing RNA content needed during rapid proliferation, and there is a marked prolongation of the regenerative process in the mouse compared to the rat (Lewan et al., 1977). However, the principal mechanisms for liver regeneration are most likely the same in the two species.

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Table 1.

Specific radioactivity of ribosomal RNA after labelling with
[methyl- ^{14}C] methionine.

Animal treatment	Time after operation (h)	Specific radioactivity of rRNA cpm/mg RNA	% of unoperated control
Unoperated control (5)	-	154 $\pm$ 7	100
Sham-operated control (5)	24	207 $\pm$ 38	134
Partial hepatectomy (6)	24	752 $\pm$ 70***	488
Sham-operated control (4)	48	191 $\pm$ 13	124
Partial hepatectomy (5)	48	387 $\pm$ 45 **	251

Mice received an i.p. injection of 6 μCi of L-[methyl- ^{14}C]methionine 180 min before liver dissection. Ribosomal RNA was isolated and the specific radioactivity was determined. For details, see Materials and Methods. The values are mean $\pm$ SEM with number of animals indicated in parenthesis. Incorporation after partial hepatectomy was compared with the values of sham-operated controls.

**) $p < 0.01$

***) $p < 0.001$

Table 2.

RNA polymerase activities in mouse liver after partial hepatectomy.

Animal treatment	Time after operation (h)	Total RNA polymerase activity		RNA polymerase I + III activity	
		pmol UMP per mg nuclear DNA	% of unoperated control	pmol UMP per mg nuclear DNA	% of unoperated control
Unoperated control	-	288 ± 26	100	175 ± 12	100
Sham-operated control	24	230 ± 19	80	150 ± 14	86
Partial hepatectomy	24	304 ± 22*	106	236 ± 19***	135
Sham-operated control	48	287 ± 23	100	177 ± 15	101
Partial hepatectomy	48	339 ± 20	118	268 ± 16***	153

Liver nuclei were isolated 24 h and 48 h after partial hepatectomy or sham operation and from unoperated controls.

After incubation with [^3H]UTP at 35° for 20 min, the rate of incorporation of [^3H]UMP into RNA by these nuclei was determined as described in Materials and Methods. Total RNA polymerase activity was measured, and RNA polymerase I plus III activity was determined in the presence of 1.4 µg/ml of α-amanitine. The results are the mean values ± SEM of 12 separate experiments, with at least 2 determinations for each preparation. The RNA polymerase activity after partial hepatectomy was compared with the values of sham-operated controls.

*) p < 0.05

***) p < 0.001

Incorporation of [14 C]orotic acid into RNA, UTP and acid soluble fraction of mouse liver.

Animal treatment	Time after operation (h)	Radioactivity ($\times 10^{-3}$) in liver metabolites						
		RNA cpm/mg RNA	% of unoperated control	UTP cmp/g liver	% of unoperated control	Acid soluble fraction cpm/g liver	% of unoperated control	
Unoperated (4) control	-	5.22 ± 0.05	100	15.9 ± 0.7	100	160 ± 3	100	
Sham-operated (5) control	24	3.41 ± 0.22	65	12.4 ± 1.8	78	120 ± 7	75	
Partial hepatectomy (4)	24	5.43** ± 0.48	104	13.6 ± 3.0	86	146 ± 13	91	
Sham-operated (5) control	48	4.40 ± 0.38	84	17.7 ± 1.8	111	148 ± 7	93	
Partial hepatectomy (5)	48	3.72 ± 0.45	71	8.3** ± 1.2	52	84*** ± 9	52	

Mice received an i.p. injection of 1.5 μ Ci of [14 C]orotic acid 120 min before liver dissection. The radioactivity of total acid soluble fraction and UTP was determined in the neutralized HClO₄-soluble fraction. The specific radioactivity of RNA was determined in the precipitated acid insoluble material after KOH hydrolysis. For details see Materials and Methods. Values are mean $\pm$ SEM with number of animals indicated in parenthesis. Incorporation after partial hepatectomy was compared with the values of sham-operated controls.

**) $p < 0.01$; ***) $p < 0.001$

MODULATION OF 5-FLUOROURACIL METABOLISM BY THYMIDINE. IN VIVO AND
IN VITRO STUDIES ON RNA-DIRECTED EFFECTS IN RAT LIVER AND HEPATOMA.

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ABSTRACT

The effects of thymidine (TdR) coadministration on the cytotoxicity and incorporation of 5-fluorouracil (5-FU) into RNA of various tissues was studied in rats bearing an ascites hepatoma (AH 130). The role of pyrimidine degradation in determining the modulating effects of TdR on the formation of FU-RNA was studied in hepatocytes and AH 130 cells in vitro. TdR (500 mg/kg) potentiated the antitumour effect of 5-FU (150 mg/kg) and also increased host toxicity as judged by changes in body weight. TdR given alone did not significantly affect tumour growth and body weight gain. Examination of the effect of TdR on the incorporation of 5-FU into RNA revealed a differential modulation of RNA-directed toxicity in different tissues. Incorporation of 5-FU into RNA in tumour and bone marrow was increased 2- and 4-fold, respectively, and was also increased to about 150% in spleen and kidney, although the increase in the latter two tissues was not statistically ascertained. In contrast, the incorporation into RNA of liver and intestinal mucosa was decreased to about 35% of the control. TdR at concentrations of 40 μ M to 40 mM progressively inhibited the degradation of 5-FU and decreased the incorporation of 5-FU into RNA of hepatocytes in vitro. In AH 130 cells in vitro TdR did not significantly influence the metabolism of 5-FU and the incorporation into RNA.

These results demonstrate that the enhanced incorporation of 5-FU into tumour RNA in vivo after pretreatment with TdR is related not to local effects on the tumour cells but rather to an increased bio-availability of the drug. Although coadministration of TdR did not selectively enhance the antitumour effect of 5-FU, a differential toxicity in host tissues was indicated by the modulated incorporation of 5-FU into RNA.

INTRODUCTION

Despite widespread clinical use the precise mechanisms by which 5-fluoro-uracil (5-FU) exerts its antiproliferative effect still remain unclear. One mode of action of 5-FU involves the conversion of 5-FU to FdUMP, which binds to thymidylate synthetase and inhibits the de novo synthesis of dTMP (1). Thereby the cellular supply of thymidine nucleotides is depleted and DNA synthesis is inhibited. It is also known that 5-FU is converted to FUTP, which then becomes incorporated into RNA (2). The incorporation into RNA leads to inhibition of the maturation of ribosomal RNA (3, 4). Furthermore, the incorporation into RNA results in impaired methylation of low molecular weight nuclear RNA (5) and decreased transcription rate of RNA (6), and incorporation into mRNA may result in miscoding during translation (7). The RNA-directed cytotoxicity of 5-FU should mainly be related to the impaired processing of rRNA. The relative roles of the DNA-directed and the RNA-directed effects of the drug have been shown to vary, depending on the biological system being studied and the experimental conditions being used. Either or both of these determinants can be of critical importance for the inhibitory actions of 5-FU on tumour proliferation and for the sensitivity of normal tissues to the drug (8).

When thymidine (TdR) is used in combination with 5-FU a reversal of the cytotoxicity of 5-FU has been found in some cultured tumour cell-lines, suggesting that inhibition of thymidylate synthetase is the major effect of 5-FU in these cells (9, 10, 11). In other experimental tumours, both in vitro and in vivo, addition of TdR leads to an enhanced cytotoxicity of 5-FU (12, 13, 14). The latter effect has been correlated with an increased incorporation of 5-FU into RNA (13, 15).

Mechanism by which TdR may enhance the incorporation of 5-FU into RNA include: (a) inhibition by dTTP of the reduction of 5-FUDP to 5-FdUDP by ribonucleotide reductase, which may lead to increased formation of 5-FUTP (12); and (b) competition by thymine for pyrimidine degrading enzymes, resulting in an increased cellular exposure to 5-FU (12, 16).

The latter effect should be pronounced in vivo since the degradation of pyrimidines is mainly localized to the liver. An increased plasma half-life of 5-FU has been demonstrated after supplementation with TdR (17, 18, 19) and would be expected to result in increased host toxicity as well as increased tumour toxicity, comparable with a higher dose of 5-FU alone. Thymidine is however reported as increasing the therapeutic index of 5-FU in some rodent tumour systems, although it has failed to do so in others (12, 20, 21, 22).

In the present work we have studied the effect of TdR on the RNA-directed action of 5-FU by measuring the incorporation of labelled 5-FU into RNA. The main objectives were: (a) to determine whether TdR potentiated the antitumour activity and host toxicity of 5-FU in rats bearing ascites hepatoma AH 130; (b) to investigate whether TdR could differentially modulate the incorporation of 5-FU into RNA of host tissues; (c) to compare the effect of TdR on the formation of FU-RNA and degradation of 5-FU in vitro in hepatocytes and AH 130 cells and to determine whether TdR, in the absence of pyrimidine degradation, may act locally on the tumour cells to increase the incorporation of 5-FU into RNA.

Results are presented demonstrating an increased antitumour activity and host toxicity of 5-FU after supplementation with TdR. An enhanced incorporation of 5-FU into tumour RNA in vivo was found to be related not to local effects on the tumour but rather to a decreased degradation of 5-FU in the liver. A differential modulation of the formation of FU-RNA was observed in normal host tissues.

MATERIALS AND METHODS

Materials

5-Fluoro [$6\text{-}^3\text{H}$]uracil (1.4 Ci/mmole) was purchased from the Radiochemical Centre, Amersham, Bucks., England; 5-Fluorouracil (25 mg/ml) was from Hoffmann-La Roche & Co. AG, Basel, Switzerland; Swim's S-77 medium was from Gibco Europe Limited, Paisley, Scotland; and thymidine was from Sigma Chemical Co., St. Louis, Mo., USA. Other chemicals used were of analytical grade.

Animals

Male Sprague Dawley rats (Anticimex, Sweden) weighing 180-200 g were used for the experiments. The animals were kept under constant conditions of light and temperature, and they had free access to food and water. The ascites hepatoma AH 130 was kindly given to us by AB Leo, Helsingborg, Sweden, and was maintained by weekly i.p. inoculation of 5×10^6 cells.

In vitro experiments

AH 130 cells were harvested on day 6 of tumour growth, washed once in Swim's S-77 medium and further diluted with the same medium. Liver cells were isolated with a collagenase perfusion method (23), washed and diluted with Swim's S-77 medium. Liver cells were incubated at a final concentration of 3×10^6 cells/ml and AH 130 cells at a concentration of 5×10^6 cells/ml. The cells were incubated in Swim's S-77 medium at 37°C in a total volume of 4 ml. After a 30 min incubation with TdR of concentrations from 0.1 $\mu\text{g/ml}$ to 10 mg/ml (0.4 μM - 40 mM), [^3H]5-FU was added (20 μCi in 50 μl Swim's S-77 medium). The lowest thymidine concentration used was within the physiological serum concentration of TdR in the rat, i.e. 0.6 - 1.3 μM (24). The final concentration of [^3H]5-FU was 3.6 μM . Incubation was carried out for another 30 min after which the

cell suspensions were centrifuged and cells and medium separately frozen.

In vivo experiments

AH 130 cells were transferred i.p. at an inoculation of 5×10^6 cells. The first 24 h after inoculation were termed day 0, the next 24 h day 1, and so on. The effect on tumour growth of 5-FU alone and 5-FU in combination with TdR was determined on day 8, after injection of the substances on day 3. 5-FU (150 mg/kg body weight, 1.2 mmole/kg) and/or TdR (500 mg/kg body weight, 2.1 mmole/kg) were injected i.p. Thymidine was administered in 1.7 ml of Ringer glucose solution 30 min before 5-FU. Control animals received Ringer glucose only.

The animals were anaesthetized by ether and killed, and the tumour was collected from the peritoneal cavity. The volume was determined, and the number of tumour cells and non-tumour cells was counted in a haematocytometer. Non-tumour cells were less than 5% of the number of tumour cells. Rats with less than 100×10^6 tumour cells were excluded. This occurred in 20% of the control rats and in 25% of the rats treated with 5-FU.

The effect of TdR on the incorporation of [^3H]5-FU into RNA of different organs was determined on day 6 of tumour growth. Thymidine (500 mg/kg) was injected i.p. in 2 ml of 0.9% NaCl. After 30 min the rats received an i.p. injection of 100 μCi [^3H]5-FU (0.36 $\mu\text{mole/kg}$). Control animals were given 2 ml of 0.9% NaCl before the injection of [^3H]5-FU. The animals were anaesthetized and killed 30 min after the injection of 5-FU. Tumour cells were collected from the peritoneal cavity, liver, spleen, kidney, intestinal mucosa and tibial bone marrow were sampled and frozen.

Tissue analysis

Tissues and isolated cells were analyzed for RNA according to the Schmidt-Tannhauser method as modified by Munro and Fleck (25). Radioactivity was measured in aliquots from the RNA-fraction.

Portions from the incubation medium were analyzed for labelled 5-FU and α -fluoro- β -alanine by chromatography on pre-coated poly(ethyleneimine)-impregnated thin-layer plates. The spots were identified by corresponding unlabelled compounds. F- β -alanine was in this study assayed as the radioactivity corresponding to β -alanine. The metabolites were eluted from the cellulose with 0.7 M MgCl_2 /20 mM Tris-HCl pH 7.4, and radioactivity was measured. Determinations of radioactivity were carried out with Insta-gel scintillation fluid (Packard) in a Packard liquid scintillation counter.

Statistical analysis of the data was performed according to Student's t-test.

RESULTS

In vitro experiments

The metabolism of 5-FU was different in hepatocytes and hepatoma cells, Table 1. In the hepatocytes the major part of the radioactivity in the medium was in the catabolic product F- β -alanine after a 30 min incubation with $[^3\text{H}]$ 5-FU. Only 6% of the acid soluble radioactivity of the medium was in 5-FU, whereas 52% was in F- β -alanine. In contrast, AH 130 cells did not degrade 5-FU to any significant extent, and no detectable radioactivity was found in F- β -alanine.

When hepatocytes were incubated with TdR (0.1 mg/ml) 30 min prior to the addition of $[^3\text{H}]$ 5-FU, the catabolic activity drastically decreased. The radioactivity of F- β -alanine was only 7% of the control, whereas the

radioactivity in 5-FU was 1085% of the control, Table 1. The specific radioactivity of RNA in hepatocytes decreased to 42% of the control. The incorporation pattern of [^3H]5-FU into the hepatoma cells was not affected by a prior incubation with 0.1 mg/ml of TdR.

To determine the threshold concentration for effect of TdR, both hepatoma cells and hepatocytes were further incubated with concentrations ranging from 0.1 $\mu\text{g/ml}$ to 10 mg/ml (0.4 μM to 40 mM). In the hepatoma cells the radioactivity of RNA in the cells and of 5-FU in the medium was practically unaffected by TdR (not shown). The specific radioactivity of RNA was 93-101% of the control value in the concentration interval 0.1 $\mu\text{g/ml}$ to 5 mg/ml of TdR. At a TdR concentration of 10 mg/ml, however, there was a slight decrease in the specific radioactivity of RNA to 76% of the control value. No radioactivity was ever demonstrated in F- β -alanine in the hepatoma cell medium.

The incorporation of [^3H]5-FU into hepatocytes was affected by rising concentrations of TdR in the way demonstrated in Figure 1. Thymidine at a concentration of 0.01 mg/ml had effect on the incorporation pattern, and with increasing concentrations of TdR the radioactivity of the catabolic product F- β -alanine of the medium steadily decreased, whereas the radioactivity of 5-FU in the medium was higher than the control value. The incorporation of [^3H]5-FU into RNA of the hepatocytes decreased with increasing concentrations of TdR, Fig. 1.

In vivo experiments

Tumour growth

The number of tumour cells on day 8 of tumour growth, after various treatments on day 3, is shown in Table 2. In the group treated with TdR only, there was no significant change in the number of tumour cells on day 8. In rats treated with 5-FU, however, there was a significant

decrease in tumour cell number to 34% of the control value. Pretreatment with TdR 30 min before 5-FU potentiated the cytotoxic effect of 5-FU and suppressed tumour growth even more effectively. The number of tumour cells on day 8 was only 19% of the control value when the rats were treated with the combination.

Incorporation of [^3H]5-FU into RNA of different organs in vivo

The effect of TdR on the incorporation of [^3H]5-FU into RNA of different organs of tumour-bearing rats is demonstrated in Table 3. There was a significant increase of the specific radioactivity of RNA of tumour and bone marrow to 213% and 396% of the control, respectively, after a prior injection of TdR. In spleen and kidney the incorporation increased by approximately 50%, but the values did not reach statistical significance. In liver and intestinal mucosa the incorporation decreased to 37% and 34%, respectively, of the control.

Body weight

Changes in body weight of tumour-bearing rats after various treatments on day 3 of tumour growth are shown in Figure 2. Inoculation of tumour cells did not affect the increase in body weight, compared to normal rats, during the first week of tumour growth. Injection of TdR (500 mg/kg) on day 3 of tumour growth did not affect the body weight increase (not shown).

The group treated with 5-FU (150 mg/kg) showed signs of toxicity resulting in a weight loss that was most accentuated on day 7. Thereafter, the rats again increased in weight. An injection of TdR 30 min prior to the treatment with 5-FU potentiated the effect of the drug, and the body weight was significantly lower than after treatment with 5-FU alone ($p < 0.001$).

DISCUSSION

Modulation of the antitumour effect of 5-FU by concurrent administration of TdR has been shown to be related to an increased incorporation of 5-FU into tumour RNA (13, 15). In our in vivo experiments we found a potentiation of the antitumour effect of 5-FU as well as increased incorporation into tumour RNA. In vitro, however, TdR did not influence the incorporation of 5-FU into tumour RNA. Accordingly, modulation by TdR of the RNA-directed effects of 5-FU on the tumour cells should be related to a decreased degradation of 5-FU by competition for pyrimidine degrading enzymes in other organs rather than to a local effect on the tumour cells.

We found extensive catabolism of 5-FU in hepatocytes *in vitro*, whereas in AH 130 cells no degradation occurred. This is in agreement with findings by Queener et al. showing that tumour cells, with the exception of well differentiated hepatomas, have a very low catabolic capacity (26). The liver is considered to be the most important site for pyrimidine catabolism in the body, and an effect on liver catabolism should therefore to a great extent influence the bio-availability of 5-FU (16).

Addition of TdR to the medium at concentrations above 4 μ M resulted in considerable inhibition of 5-FU degradation in hepatocytes whereas no effect on the metabolism of 5-FU was found in tumour cells. As a consequence in vivo of an inhibited degradation of 5-FU by TdR, one would expect an increased bio-availability of 5-FU. In fact, TdR has been shown to increase plasma half-life of 5-FU (17, 18, 19) and reduce the amounts of catabolic products in serum (27).

An increased availability of 5-FU and prolonged cellular exposure to the drug is considered to be the main mechanism for the observed increase in potency and incorporation into tumour RNA in vivo (15). However, in some experimental cell systems in vitro, a potentiation of the incorporation of 5-FU into RNA has been observed after addition of TdR (14, 28).

The biochemical basis for this effect might be mediated by the inhibition by dTTP of ribonucleotide reductase reduction of 5-FUDP to 5-FdUDP (12) thus promoting the formation of 5-FUTP. Such a mechanism does not seem to operate in AH 130 cells, since no effect of TdR on the incorporation of 5-FU into RNA was observed in vitro. The decreased incorporation of 5-FU into RNA of hepatocytes after addition of TdR was probably related to overloading of the pyrimidine degrading enzymes. A decreased degradation of endogenous uracil may lead to expansion of uracil nucleotide pools, and consequently 5-FU has to compete with more endogenous precursors for incorporation into RNA.

The combination with TdR markedly increased the effects of 5-FU in vivo. A potentiation of the incorporation of 5-FU into tumour RNA was observed as well as a reduction in tumour growth. Host toxicity was also increased, as judged by the body weight decrease. Moreover, the increased incorporation of 5-FU into bone marrow RNA indicated an increased toxicity in this organ. On the other hand, the incorporation into RNA of intestinal mucosa was decreased.

Whether modulation of RNA-directed effects of 5-FU is the sole determinant of the altered cytotoxicity obtained by pretreatment with TdR is not known. Inhibition of DNA-synthesis through 5-FdUMP inhibition of thymidylate synthetase is undoubtedly an important mechanism for the toxicity of 5-FU. Although by addition of TdR the cells may overcome this block (21), the alteration of 5-FU pharmacokinetics may affect the DNA-directed effects when TdR has been cleared from the circulation. Moreover, through the action of thymidine phosphorylase, deoxyribose-1-P released from TdR can be utilized in the conversion of 5-FU to 5-FUdR (19). Increased levels of circulating 5-FUdR have been observed in patients given 5-FU in combination with TdR (18, 19). Considering that

5-FuDR is more cytotoxic in vitro than 5-FU (29), an increased conversion of 5-FU to 5-FuDR may have profound therapeutic and toxicological consequences. The resultant toxicity of 5-FU in different tissues after modulation by TdR should thus be dependent on the relative importance of RNA- and DNA-directed actions of 5-FU in the different tissues and would be expected to differ between tissues and species according to the relevant enzyme activities.

Thymidine is reported to increase the therapeutic index of 5-FU in some rodent tumour systems but has failed to do so in a number of others (12,20, 21, 22). Clinical trials with 5-FU and TdR in combination have not appeared to improve the therapeutic index of 5-FU (15). Although antitumour activity is potentiated, marrow toxicity and neurotoxicity are concomitantly increased (17, 30). In contrast to the increased marrow toxicity, gastrointestinal toxicity is reported as not being increased (15, 30).

The fact that the intestinal toxicity of 5-FU is not potentiated by TdR may possibly be related to a decreased incorporation of 5-FU into RNA as found in our studies on rat intestinal mucosa. Based on the concept of the importance of RNA-directed effects for the cytotoxicity of 5-FU, this may provide a basis for an improved therapeutic index of 5-FU by development of multiagent treatment protocols.

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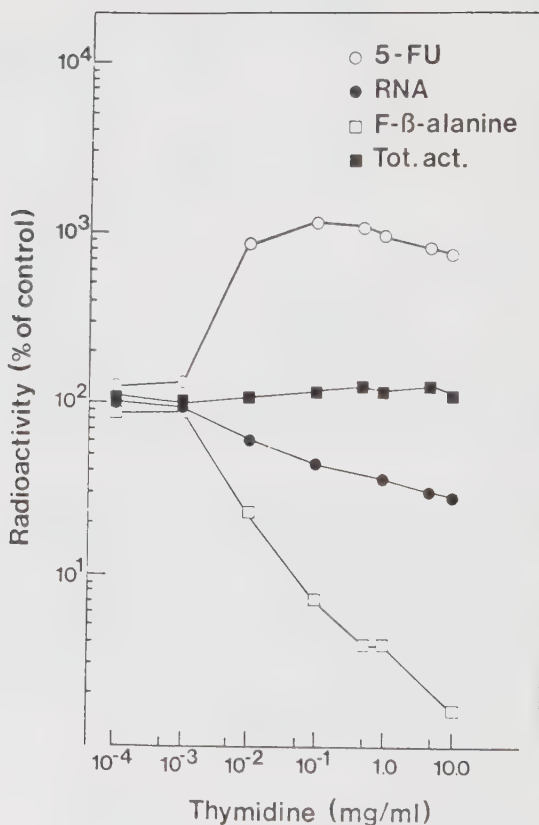
Table 1. Metabolism of [^3H] 5-FU in hepatocytes and hepatoma cells in vitro.
Effect of thymidine (0.1 mg/ml).

	Conditions of incubation	Radioactivity (cpm $\times 10^{-3}$ /ml) in medium			RNA specific radioactivity (cpm/mg RNA)
		5-FU	F- β -alanine	Total acid soluble fraction	
Hepatocytes (3)	5-FU	76 (100%) ± 12	668 (100%) ± 38	1281 (100%) ± 54	31 632 (100%) ± 5923
	TdR+5-FU	828*** (1085%) ± 44	46*** (7%) ± 3	1549* (121%) ± 60	13 241* (42%) ± 412
Hepatoma cells (2)	5-FU	840 (100%)	0	1379 (100%)	85 770 (100%)
	TdR+5-FU	865 (103%)	0	1348 (98%)	81 481 (95%)

Hepatocytes and AH 130 cells were incubated with [^3H] 5-FU (3.6 μM , 1.4 Ci/mmol) for 30 min. Thymidine (TdR, 0.1 mg/ml) was added 30 min prior to [^3H] 5-FU. The radioactivity of the total acid soluble fraction, of 5-FU and of F- β -alanine of the medium was analyzed as well as the specific radioactivity of RNA of the cells. In each experiment the incubations were carried out in duplicate. Results are mean $\pm$ SEM from 3 independent experiments with hepatocytes and 2 experiments with hepatoma cells. Figures in parenthesis are % of control value (5-FU only).

*) $p < 0.05$; ***) $p < 0.001$

Figure 1. Effect of thymidine on the metabolism of 5-FU in hepatocytes in vitro.



Hepatocytes were incubated with [³H]5-FU (3.6 μM, 1.4 Ci/mmol) for 30 min. Thymidine at increasing concentrations was added 30 min prior to 5-FU. The radioactivity of the total acid soluble fraction, of 5-FU and of F-β-alanine of the medium was analyzed as well as the specific radioactivity of RNA of the cells. Radioactivity is expressed as % of control value (5-FU only). In each experiment the incubations were carried out in duplicate. Results are mean from three separate experiments.

Table 2. Effect on tumour growth of 5-FU alone and of 5-FU in combination with thymidine.

Treatment on day 3	Number of tumour cells $\times 10^{-6}$	% of control	Number of animals
Control	4 134 $\pm$ 160	100	6
TdR	3 832 $\pm$ 258	93	5
5-FU	1 411 ^a $\pm$ 158	34	5
TdR + 5-FU	770 ^{a,b} $\pm$ 166	19	7

Number of cells of hepatoma AH 130 were determined on day 8 of tumour growth after various treatments on day 3. Thymidine (TdR, 500 mg/kg) was given i.p. in 1.7 ml of Ringer glucose 30 min before 5-FU (150 mg/kg, i.p.). Control rats received Ringer glucose only. Results are mean $\pm$ SEM.

a) significantly different from control and TdR
p < 0.001

b) significantly different from 5-FU
p < 0.05

Table 3. Effects of thymidine on the incorporation of [^3H]5-FU into RNA of different organs of tumour bearing rats.

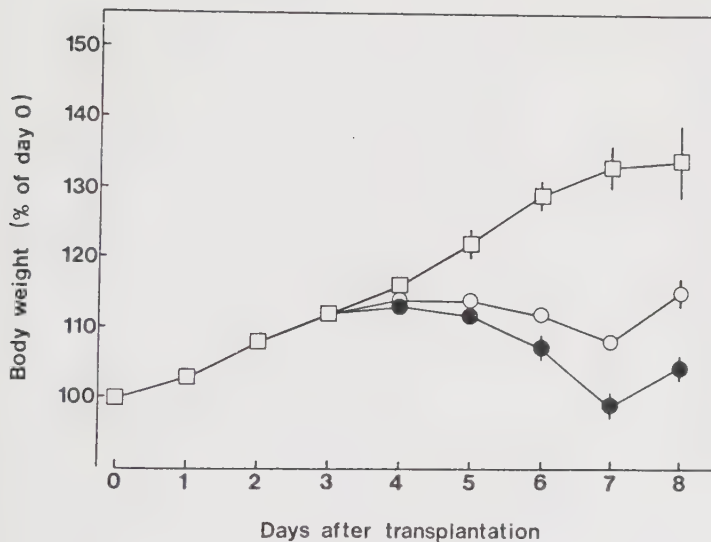
Organ	Specific radioactivity of RNA (cpm/mg RNA)					
	NaCl			TdR		
Tumour	13 525 ± 636	(100%)	(5)	28 850** ± 4081	(213%)	(4)
Liver	1832 ± 323	(100%)	(5)	686* ± 64	(37%)	(4)
Spleen	1414 ± 337	(100%)	(4)	2185 ± 271	(155%)	(3)
Kidney	1406 ± 269	(100%)	(5)	1963 ± 351	(140%)	(4)
Intestinal mucosa	3407 ± 742	(100%)	(5)	1158* ± 160	(34%)	(4)
Bone marrow	439 ± 29	(100%)	(5)	1739*** ± 172	(396%)	(4)

On day 6 of tumour growth the animals were given an i.p. injection of TdR (500 mg/kg) or NaCl (0.9%), and 30 min later they received [^3H]5-FU (100 μCi , 0.36 $\mu\text{mole/kg}$). The animals were killed 30 min after the administration of 5-FU. The specific radioactivity of RNA in different organs was determined. Results are mean $\pm$ SEM of duplicate samples from three to five animals, shown in parenthesis.

*) $p < 0.05$; **) $p < 0.01$; ***) $p < 0.001$

Figure 2.

Effect on body weight of 5-FU alone and 5-FU in combination with TdR.



Body weight of tumour-bearing rats (□), (4); Tumour-bearing rats treated with 5-FU (150 mg/kg) on day 3 (○), (9); Tumour-bearing rats treated with TdR (500 mg/kg) and 5-FU (150 mg/kg) on day 3 (●), (12). Results are mean $\pm$ SEM with number of animals indicated in parenthesis.

